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(71) Applicant (for all designated States except US): **ACAMBIS RESEARCH LIMITED** [GB/GB]; Peterhouse Technology Park, 100 Fulbourn Road, Cambridge CB1 9PT (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **TURNER, Arthur, Keith** [GB/GB]; Acambis Research Limited, Peterhouse Technology Park, 100 Fulbourn Road, Cambridge CB1 9PT (GB). **GREENWOOD, Judith** [GB/GB]; Acambis Research Limited, Peterhouse Technology Park, 100 Fulbourn Road, Cambridge CB1 9PT (GB). **STEPHENS, Jonathan, Clive** [GB/GB]; Acambis Research Limited, Peterhouse Technology Park, 100 Fulbourn Road, Cambridge CB1 9PT (GB). **BEAVIS, Juliet, Claire** [GB/GB]; Acambis Research Limited, Peterhouse Technology Park,

100 Fulbourn Road, Cambridge CB1 9PT (GB). **DARSELEY, Michael, James** [GB/GB]; Acambis Research Limited, Peterhouse Technology Park, 100 Fulbourn Road, Cambridge CB1 9PT (GB).

(74) Agents: **CAMPBELL, Patrick, John, Henry** et al.; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5JJ (GB).

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(54) Title: ATTENUATED BACTERIA USEFUL IN VACCINES

(57) Abstract: The invention provides strains of bacteria, especially enterotoxigenic *E. coli*, attenuated by mutations in the genes encoding enterotoxins (LT, ST, EAST1) and optionally further attenuated by deletion of additional chromosomal genes. In addition the invention provides strains of attenuated bacteria expressing immunogenic but non-toxic variants of one or more of these enterotoxins. These bacteria are useful as a vaccine against diarrhoeal disease.



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## ATTENUATED BACTERIA USEFUL IN VACCINES

The invention relates to attenuated bacteria useful in vaccines.

### Background to the invention

5 The principle behind vaccination is to induce an immune response in the recipient, thus providing protection against subsequent challenge with a pathogen. This may be achieved by inoculation with a live attenuated strain of the pathogen, i.e. a strain having reduced virulence such that it does not cause the disease caused by the virulent pathogen while still stimulating a broad immune response.

10

Using modern genetic techniques, it is now possible to construct site-directed attenuated bacterial strains in which stable attenuating deletions have been created. A number of site-directed mutants of *Salmonella* have been created using this type of technology (2, 7, 9, 14, 19, 35, 36, 37). Mutations in a large number of genes have  
15 been reported to be attenuating, including the *aro* genes (e.g. *aroA*, *aroC*, *aroD* and *aroE* (15, 18)), *pur*, *htrA* (4), *ompR*, *ompF*, *ompC* (2), *galE* (14), *cya*, *crp* (7), *phoP* (13, 19), *rfaY* (48), *dksA* (48), *hupA* (48), *sipC* (48) and *clpB* (48).

One class of bacterium that has been attenuated by such modern genetic techniques is  
20 enterotoxigenic *Escherichia coli* (ETEC), which causes diarrhoea. The virulence of (ETEC) strains depends on their expression of fimbrial colonization factor antigens (CFAs) which allow them to attach to and colonize the mucosal surface of the small intestine of their host species. Human adapted ETEC strains express a number of CFAs, the most frequently occurring of which are CFA/I, CFA/II (comprising CS3  
25 expressed with either CS1 or CS2) and CFA/IV (comprising CS6 expressed alone or with either CS4 or CS5). Depending on the geographic location, CFA/I, CFA/II and CFA/IV account for between 50% and 80% of ETEC strains. Many other CFAs have been described, but each of them is found in only a small proportion of ETEC strains (33). Evidence indicates that anti-CFA immune responses are important for  
30 protection against ETEC disease (6, 24, 28, 29, 30).

Colonization of the small intestine is accompanied by the secretion of enterotoxins. Two types of enterotoxins have been identified in ETEC strains, the heat labile toxin (LT) and the heat stable toxin (ST). LT is highly homologous in structure to the cholera toxin, a multi-subunit protein of the form AB<sub>5</sub>. The A subunit is the active component of the toxin, which functions to increase the activity of adenylate cyclase. This is delivered into host cells by the B subunits, which bind to gangliosides on the cell surface. ST is a small (19 amino acid) non-immunogenic polypeptide that has guanylate cyclase stimulating activity. In addition, it has been demonstrated recently that a large proportion of ETEC strains also produce EAST1, a heat-stable toxin similar in size and mode of action to ST but different in sequence, which was originally identified in enteroaggregative *E. coli* strains (34).

It has been proposed that derivatives of ETEC strains, which have lost the ability to produce toxins, may be effective live vaccines against virulent isolates. A derivative of a wild-type ETEC strain, E1392/75, that has spontaneously lost the ST and LT activities but which continues to express CFA/II was identified and designated E1392/75-2A (5). In human volunteer studies, oral vaccination with  $2 \times 10^{10}$  cfu E1392/75-2A gave 75% protection against challenge with a toxin-expressing ETEC from a different serotype but which expressed the same CFAs (reviewed by (30)). However, approximately 15% of vaccinees experienced mild diarrhoea as a side effect of the vaccine. It was concluded that further attenuation of this strain was required before it could be considered for use as a live vaccine against ETEC infections.

Two derivatives of E1392/75-2A were generated by targeted deletion of potential attenuating genes and evaluated in clinical trials (32, 38). It was demonstrated that both of the derivatives (PTL002,  $\Delta aroC/\Delta ompR$ , and PTL003,  $\Delta aroC/\Delta ompC/\Delta ompF$ ) were attenuated when compared to the parent strain and caused no clinical symptoms in volunteers who ingested up to  $5 \times 10^9$  cfu of freshly harvested live organisms. All volunteers receiving the maximum dose of these candidate vaccines generated specific immune responses against the CFA/II antigen expressed by the strains.

### **Summary of the Invention**

An effective vaccine against ETEC must immunize against CFA/I, CFA/II and CFA/IV as a minimum, and therefore attenuated strains expressing all of these antigens are required. Thus, it is required that the genes expressing the toxins LT,  
5 ST and EAST1 are inactivated or deleted from strains expressing all of these CFAs. Toxin minus strains have previously been suggested as a starting point for developing a live attenuated multi-strain vaccine against ETEC (Chatfield, 38). There was, however, no explanation in Chatfield as to how such strains might be generated.

10 We have now found that there are particular difficulties associated with the generation of a strain expressing CFA/I or a strain expressing CS5 and CS6 from which the toxin genes, especially the ST gene, have been deleted. We have devised a novel strategy and suicide vector for overcoming these difficulties and producing toxin minus forms of these strains.

15

Without wishing to be bound by this theory, we believe that the reason that ST minus forms of strains expressing CFA/I or CS5 and CS6 were difficult to generate was that the CFA/CS genes are closely linked to the ST gene and are on the same plasmid. In a global review of epidemiological studies in which sufficient data had been  
20 collected (33) it is reported that of 204 CFA/I expressing strains, all 204 of them expressed ST, either alone (149/204) or in combination with LT (55/204). Further, more recent studies have confirmed this finding, e.g. Qadri et al (22), where all of 87 CFA/I expressing strains isolated in a two year period in Bangladesh expressed ST, either alone or in combination with LT. No strains were identified which expressed  
25 CFA/I and LT alone, suggesting an extremely tight genetic linkage between the ST and CFA/I loci. Numerous scientific papers document the close linkage between CFA/I and ST genes in ETEC strains, to the extent that whenever an effort has been made to derive a strain which has lost one of these loci, the other has always been lost concurrently. In no instance of which we are aware has it been possible to  
30 separate the two loci by deletion or inactivation of the ST gene and produce a strain that still expresses CFA/I (42-46).

The invention provides a bacterial cell which expresses colonization factor antigen CFA/I from a native plasmid but does not express heat stable toxin (ST). The invention also provides a bacterial cell which expresses colonization factor antigen CS5 from a native plasmid and/or expresses colonization factor antigen CS6 from a native plasmid, but does not express heat stable toxin (ST). The LT gene and the EAST1 gene may also be deleted or inactivated in the cells of the invention. The cells generally contain further attenuating mutations, such as mutations in each of the *aroC*, *ompF* and *ompC* genes, in order to make them acceptable for use in vaccines.

The cells of the invention may be genetically engineered to express a heterologous antigen, such as a non-toxic component or form of LT, or a colonization factor antigen (CFA). Such cells induce an immune response against the heterologous antigen as well as the native antigens and hence improve the protection provided by a vaccine.

The invention includes a vaccine against diarrhoea containing the cells of the invention. Preferably, the vaccine includes a blend of different cells which between them carry all the most common CFAs, namely CFA/I, CFA/II and CFA/IV.

Furthermore, the invention provides a suicide vector and a method which makes possible the reliable and rapid isolation of the cells of the invention and other bacterial cells containing deleted, inactivated or replaced genes. The vector represents an improvement over known suicide vectors in that it allows more specific and more reliable targeting than known vectors. The vector is less than 5 kb in size (e.g. from 2.5 to 5 kb or from 2.5 to 4 kb) and comprises the *sacB* region which codes for a product that is toxic to bacteria when grown on sucrose, in which region the IS1 insertion sequence is deleted or inactivated. The small size of the vector and the absence of the IS1 insertion sequence help to prevent the vector from targeting to the wrong place in the cellular DNA.

## **Detailed Description of the Invention**

### **Bacteria useful in the Invention**

The bacterial cells of the invention are generally derived from enterotoxigenic *E. coli* (ETEC) cells by deletion or inactivation of the ST gene and optionally other toxin genes. As mentioned above, ETEC is a class of *E. coli* that cause diarrhoea. They colonise the small intestine. They can be isolated from human clinical samples, typically stools produced whilst suffering from diarrhoea. A standard ETEC strain is H10407, deposited at the ATCC under catalogue #35401.

10

Infections of ETEC are the single most frequent cause of travellers diarrhoea, causing 3-9 million cases per year amongst visitors to developing countries. In endemic areas, ETEC infections are an important cause of dehydrating diarrhoea in infants and young children, resulting in up to 400,000 deaths a year, predominantly in this age group. In developing countries, the incidence of ETEC infections leading to clinical disease decreases with age, indicating that immunity to ETEC infection can be acquired. In contrast, naive adults from industrialized countries who visit endemic areas are highly susceptible to ETEC infections. However, with prolonged or repeated visits to endemic areas susceptibility to ETEC infections diminishes, suggesting that a live attenuated approach to ETEC vaccination may prove successful.

20

A vaccine to protect against ETEC diarrhoea in humans must provide protection against the seven major colonization factors and, as a minimum, the heat labile toxin (LT) to ensure that protection against different strains is obtained. In order to achieve this, the same attenuations could be made in a range of different ETEC strains, each with a different colonization factor. This would involve deleting the toxins from all such strains. The present invention provides a panel of suitable strains from which all toxin genes have been completely deleted which can provide the starting point for the generation of a multi-strain vaccine. Alternatively, it may be

30

possible to express multiple colonization factors in a smaller number of strains from which the toxins have been similarly deleted.

- 5 Toxin-deleted strains of the present invention were derived from wild-type clinical isolates obtained from a long-term epidemiological study carried out in Egypt by scientists at the US Navy NAMRU3 facility in Cairo. A list of the strains provided is given in the following table.

Strain	Code	Phenotype	CFA	LT	ST	EAST1
WS-1858B	A	O71:H-	CFA/I	-	+	+
WS-4437A	B	O128:H12	CFA/I	-	+	-
WS-6117A	C	O153:H45	CFA/I	-	+	+
WS-2560B	D	O25:H-	CS4, CS6	+	+	+
WS-2773E	E	O39:H12	CS5, CS6	+	+	+
WS-4150D	F	O6:H16	CS2, CS3	+	-	-
WS-6170A	G	O17:H18	CS2, CS3	-	+	-
WS-3504D	H	O141:H5	CS2, CS3	+	+	+
WS-3517A	I	O6:H-	CS2, CS3	-	+	+
WS-2252A	J	O15:H18	CS4, CS6	+	+	+
WS-2511A	K	O4:H-	CS4, CS6	-	+	+
WS-2556A	L	O6:H1	CS4, CS6	-	+	+
WS-4046A	M	O39:H-	None identified	+	-	N.D.

- 10 It will be clear to those skilled in the art that other strains may be equally suitable as a starting point for the generation of a toxin-deleted, attenuated multi-strain vaccine.

- The strains with the codes A, B, E, H & J were attenuated by the specific removal of all known toxin genes and then further manipulated, as described in the  
 15 accompanying Examples. Resulting toxin-minus strains and strain PTL003 described above were deposited by Acambis Research Limited of Peterhouse Technology

- Park, 100 Fulbourn Road, Cambridge, CB1 9PT, United Kingdom with the European Collection of Cell Cultures (ECACC), CAMR, Salisbury, Wiltshire SP4 OJG, United Kingdom on 3<sup>rd</sup> September 2001 (accession numbers 010903\*\*) or 29<sup>th</sup> August 2002 (accession numbers 020829\*\*) in accordance with the Budapest Treaty. The
- 5 characteristics of the strains and the accession numbers of those which were deposited are as follows:



Strain	Parent Strain	Accession number	LPS:flagellin	Antibiotic Resistance	CS proteins	Regulator	Toxin Genes	Example
E1392/75-2A PTL003	E1392/75 E1392/75-2A	N/A 01090302 (submitted as ACM2005)	O6:H16 O6:H16	Strep Strep	CS1 CS3 CS1 CS3	<i>rns</i> <i>rns</i>	None None	
ACAM2008	PTL003	02082965	O6:H16	None	CS1 CS3	<i>rns</i>	None	
WS-2773E (strain E) WS-2773E-Tox minus	N/A WS-2773E	N/A 01090305 (submitted as ACM2002)	O39:H12 O39:H12	None None	CS5 CS6 CS5 CS6	? <i>csvR</i> ? <i>csvR</i>	ST EAST LT None	3
ACAM2006 ACAM2012*	WS-2773E-Tox minus ACAM2006	N/A 02082968	O39:H12 O39:H12	None None	CS5 CS6 CS5 CS6	? <i>csvR</i> ? <i>csvR</i>	None None	
WS-3504D (strain H) WS-3504D-Tox minus	N/A WS-3504D	N/A 01090304 (submitted as ACM2003)	O141:H5 O141:H5	Amp Amp	CS2 CS3 CS2 CS3	<i>rns</i> <i>rns</i>	EAST None	4
ACAM2007	WS-3504D-Tox minus	02082964	O141:H5	None	CS2 CS3	<i>rns</i>	None	
WS-1858B (Strain A) WS-1858B-Tox minus	N/A WS-1858B	N/A N/A	O71:H- O71:H-	Amp/Tmp/Smz Amp/Tmp/Smz	CFA/I CFA/I	<i>rns</i> <i>rns</i>	ST EAST None	2
ACAM2010	WS-1858B-Tox minus	02082967	O71:H-	None	CFA/I	<i>rns</i>	None	
WS-2252A (Strain J) WS-2252A-Tox minus	N/A WS-2252A	N/A 01090306 (submitted as ACM2004)	O15:H18 O15:H18	None None	CS4 CS6 CS4 CS6	<i>cfad</i> <i>cfad</i>	ST EAST LT None	4
ACAM2009	WS-2252A-Tox minus	02082966	O15:H18	None	CS4 CS6	<i>cfad</i>	None	
WS-2511A (Strain K) Strain K	N/A WS-2511A-Tox minus	N/A N/A	O4:H- O4:H-	None None	CS4 CS6 CS6	<i>cfad</i> <i>cfad</i>	ST EAST x 2 ST EAST x 2	N/A

\* ACAM2006 contains a lysogenic phage in its chromosome – ACAM2012 is a derivative of ACAM2006 from which a large part of the genome, including several genes critical for phage assembly, have been deleted.

In addition to the strains in the above table, a toxin minus derivative of strain B described in Example 2 below was deposited under accession number 01090303.

The invention includes "descendents" of these deposited cells. A "descendent" is  
5 any cell derived from a deposited cell. The descendents of a deposited cell include cells with one or more further attenuating mutations, such as the mutations described below. Descendents also include cells which have been engineered to express heterologous antigens, such as the heterologous antigens described below.

10 Although the bacteria of the invention are generally *E. coli* bacteria, other types of bacteria may be used. A plasmid in accordance with the invention may be constructed by deleting or inactivating the ST gene in a plasmid native to enterotoxigenic *E. coli*, and then transferring the resulting plasmid to another bacterium. In this way, the other bacterium may be made to carry the CFA/I antigen  
15 or the CS5 and CS6 antigens.

The bacteria that are used to make the vaccines of the invention are generally those that infect by the oral route. The bacteria may be those that invade and grow within eukaryotic cells and/or colonize mucosal surfaces. The bacteria are generally gram  
20 negative but in some embodiments gram positive bacteria may be used. The bacteria are generally pathogens.

The bacteria used may be from the genus *Escherichia*, *Salmonella*, *Shigella* or *Vibrio*.

25 Other than *E. coli*, examples of the species of bacteria that can be used in the invention are *Salmonella typhimurium* - the cause of salmonellosis in several animal species; *Salmonella typhi* - the cause of human typhoid; *Salmonella enteritidis* - a cause of food poisoning in humans; *Salmonella choleraesuis* - a cause of  
30 salmonellosis in pigs; and *Salmonella dublin* - a cause of both a systemic and diarrhoeal disease in cattle, especially of new-born calves.

- Strains of *E.coli* and *Salmonella* are particularly useful in the invention. *Salmonella* are potent immunogens and are able to stimulate systemic and local cellular and antibody responses. In particular an attenuated strain of *Salmonella typhi* is preferred for use in the invention. Preferred *Salmonella typhi* strains for use in the present invention include CVD908-htrA ( $\Delta aroC \Delta aroD \Delta htrA$ ) and CVD908 ( $\Delta aroC \Delta aroD$ ) (49). Strains of *E.coli* other than ETEC that may be used include enteropathogenic *E.coli* (EPEC), enteroinvasive *E.coli* (EIEC) and enterohemorrhagic *E.coli* (EHEC).
- As used herein, references to a "native" plasmid which expresses CFA/I or CS5/6 mean a plasmid which exists in wild-type cells, for example ETEC cells isolated from a person with diarrhoea. They exclude a plasmid constructed in the laboratory for expression of CFA/I or CS5/6. In a cells of the invention, the ST gene in the native plasmid is deleted or inactivated. However, the CFA/I or CS5/6 gene in the plasmid is functional, i.e. it expresses CFA/I or CS5/6.

#### Further mutating the bacteria

- In order for the bacteria to be used in a vaccine, they must generally be further attenuated. The attenuation may, for example, be brought about by deleting or inactivating one or more of the following genes: *aroA*, *aroC*, *aroD*, *aroE*, *pur*, *htrA*, *ompC*, *ompF*, *ompR*, *cya*, *crp*, *phoP*, *surA*, *rfaY*, *dksA*, *hupA*, *sipC* and *clpB*. Preferred combinations of genes include:

- at least one *aro* gene (e.g. *aroA*, *aroC*, *aroD* or *aroE*) and at least one *omp* gene (e.g. *ompC*, *ompF* or *ompR*);
- at least one *aro* gene (e.g. *aroA*, *aroC*, *aroD* or *aroE*) and the *htrA* gene;
- *aroC*, *ompF* and *ompC*.

- The further attenuating mutations may be introduced using the suicide vector and methods of the invention or by methods known to those skilled in the art (see ref. 25). Appropriate known methods include cloning the DNA sequence of the wild-type

gene into a vector, e.g. a plasmid, and inserting a selectable marker into the cloned DNA sequence or deleting a part of the DNA sequence, resulting in its inactivation. A deletion may be introduced by, for example, cutting the DNA sequence using restriction enzymes that cut at two points in or just outside the coding sequence and ligating together the two ends in the remaining sequence. Alternatively, and more usually now, a mutant allele in which the flanking regions of a target gene are amplified separately and linked directly together in a separate overlap PCR reaction, with omission of the intervening target sequence, can be constructed (32). A plasmid carrying the mutated DNA sequence can be transformed into the bacterium by known techniques such as electroporation and conjugation. It is then possible by suitable selection to identify a mutant wherein the inactivated DNA sequence has recombined into the chromosome of the bacterium and the wild-type DNA sequence has been rendered non-functional by homologous recombination.

Furthermore, the antibiotic resistance genes must generally be removed from the bacteria before they are used in a vaccine. Bacteria isolated from the wild often contain antibiotic resistance genes, such as resistance genes against ampicillin, streptomycin, sulphmethoxazole, kanamycin, trimetheprim and tetracyclin. These genes can be removed using the suicide vector and methods of the invention or by methods known to those skilled in the art.

### **The nature of the mutations**

The mutations introduced into the bacterial vaccine to prevent expression of enterotoxins or other virulence genes delete or inactivate the gene. They generally knock-out the function of the gene completely. This may be achieved either by abolishing synthesis of any polypeptide at all from the gene or by making a mutation that results in synthesis of non-functional polypeptide. In order to abolish synthesis of polypeptide, either the entire gene or its 5'-end may be deleted. A deletion or insertion within the coding sequence of a gene may be used to create a gene that synthesises only non-functional polypeptide (e.g. polypeptide that contains only the

N-terminal sequence of the wild-type protein). In the case of a toxin gene, the mutation may render the gene product non-toxic.

5 The mutations are generally non-reverting mutations. These are mutations that show essentially no reversion back to the wild-type when the bacterium is used as a vaccine. Such mutations include insertions and deletions. Insertions and deletions are preferably large, typically at least 10 nucleotides in length up to the length of the entire gene or coding sequence, for example from 10 to 600 nucleotides. Preferably, the whole coding sequence or whole gene is deleted.

10

The mutations are typically site-directed. They may be specific or selective to the toxin gene or other virulence factor gene. In the case of deleting or inactivating the ST gene in a CFA/I or CS5/CS6 strain, the mutation must specifically target the ST gene without deleting or inactivating the (closely-linked) CFA/I gene, CS5 gene or  
15 CS6 gene.

### Expression of heterologous antigens

An attenuated bacterium of the invention may be genetically engineered to express an antigen that is not expressed by the native bacterium (a "heterologous antigen"),  
20 so that the attenuated bacterium acts as a carrier of the heterologous antigen. In the case that the bacterium is an ETEC bacterium, the antigen may be from another species or from another strain of ETEC, so that the vaccine provides protection against the other species or strain. Furthermore, the bacterium may be engineered to express more than one heterologous antigen, in which case the heterologous antigens  
25 may be from the same or different species or strains.

The heterologous antigen may be a complete protein, a part of a protein containing an epitope or a fusion protein. The antigen may be from another bacterium, a virus, a yeast or a fungus. More especially, the antigenic sequence may be from a pathogenic

strain of *E.coli* (e.g. ETEC). Useful antigens include ETEC colonization factor antigens, non-toxic components or non-toxic mutants of *E.coli* LT (e.g. the B subunit and mutants of the A subunit), LT-ST fusion proteins and cholera toxin B subunit (CT-B) (50-55).

5

The ETEC CFAs and components thereof are prime candidates for expression as heterologous antigens. To instigate diarrhoeal disease, pathogenic strains of ETEC must be able to colonize the intestine and elaborate enterotoxins. For most strains of ETEC, CFAs that are responsible for adhesion to the intestinal mucosa have been  
10 identified. In almost all cases CFAs are expressed as fimbriae on the outer surface of the bacteria. A large number of CFAs have been identified, the most prevalent being CFA/I, CFA/II (includes CS1, CS2, CS3) and CFA/IV (includes CS4, CS5, CS6). Additional antigens include CS17, CS7, CS9, CS14, CS12, PCFO159, PCFO166.

15 The DNA encoding the heterologous antigen may be expressed from a promoter that is active *in vivo*. Promoters that have been shown to work well are the *nirB* promoter (10, 39), the *htrA* promoter (10), the *pagC* promoter (57) and the *ssaH* promoter (58). For expression of the ETEC colonization factor antigens or derivatives of LT, CT or ST, the wild-type promoters could be used.

20

A DNA construct comprising the promoter operably linked to DNA encoding the heterologous antigen may be made and transformed into the attenuated bacterium using conventional techniques. Transformants containing the DNA construct may be selected, for example by screening for a selectable marker on the construct. Bacteria  
25 containing the construct may be grown *in vitro* before being formulated for administration to the host for vaccination purposes.

### Plasmid stabilisation

In order to prevent loss of the plasmid expressing the heterologous antigen or of a native plasmid, an element may be added to the plasmid which enhances its stability. It is generally the case that the plasmids found in ETEC strains encoding the various colonization factor antigens are low copy number and stable enough to ensure their maintenance over many generations in the absence of specific selection mechanisms. However, following manipulation of these plasmids in accordance with the invention, for example to delete genes for toxins such as ST or other virulence determinants, these stable properties might be impaired. This problem may be alleviated by employing methods for improvement of plasmid stability.

There are a number of "toxin/antitoxin" plasmid stability determining systems known, for example *parDE* (23) from plasmid RP4 (1), and *hok/sok* (also known as *parB* from plasmid R1 or *pndAB* from plasmid R483 (11, 12)) which could be used to do this. These systems encode two functions: firstly a toxic entity that would kill cells in which it is expressed, which has a long biological half-life, and secondly an antitoxic entity that prevents this killing but has a short biological half-life. In the event that a plasmid encoding these functions is segregated during division the daughter cell which does not contain the plasmid exhausts its supply of antitoxin and is killed by the more persistent toxin moiety. Thus, only cells that continue to harbour the plasmid are maintained in the growing population.

Another system that may be used to enhance the stability of a plasmid in accordance with the invention is a multimer resolution system. Multimer resolution systems confer stability by resolving plasmid multimers into single plasmid copies and hence decreasing the chance of plasmid free daughter cells being generated by random segregation at cell division. A number of site-specific recombination systems which act to resolve plasmid multimers into monomers have been identified. In accordance with such a system, the plasmid to be stabilised contains a recognition site for a site-specific recombinase and the host cell contains a DNA sequence encoding a site-specific recombinase. The recombinase acts on the recognition site and thereby directs proper segregation of the plasmid during cell division. The recombinase may be encoded on the plasmid to be stabilised or in the chromosome of the host cell.

The recombinase is generally a resolvase. Examples of resolvases which may be used in the invention include the Cre recombinase of plasmid P1, the *E.coli* XerC (ArgR) protein, the D protein recombinase of plasmid F, the ParA recombinases of plasmids RP4 and RK2, the site-specific recombinase of plasmid R1, resolvases encoded by  
5 the Tn3-like transposable genetic elements and the Rsd resolvase from the *Salmonella dublin* virulence plasmid.

The recognition elements which may be used in the present invention include those for the above recombinases. Any recognition element recognised by the site-specific  
10 recombinase employed may be used. Suitable recognition elements include those sites recognised by the XerC site-specific recombinase, such as the *cer* site of plasmid ColE1 and the similar *ckr* site of plasmid ColK (59), the *psi* site of plasmid pSC101 and the *cer* like site of plasmid pHS-2 from *Shigella flexneri*. Other recognition elements which may be used include the *crs* site from the *Salmonella*  
15 *dublin* virulence plasmid, the *loxP* site of plasmid P1, the *rfs* site of the F plasmid and the *res* site of the Tn3-like transposable genetic element

In a particularly preferred embodiment of the invention, the recombinase is the Rsd resolvase which acts via the *crs* recognition element. The Rsd/*crs* system is  
20 described in detail in our copending application, UK Patent Application No. 0024203.2.

### Formulation of vaccines

The invention provides a vaccine against diarrhoea comprising an *E.coli* cell of the  
25 invention and a pharmaceutically acceptable carrier or diluent. Generally, the vaccine includes a blend of different toxin minus cells (e.g. 2, 3, 4, 5 or 6 cells) which between them carry all the most common CFAs. For example, the vaccine may contain five different strains of toxin minus cell as follows:

- 30 (i) a cell which expresses CFA/I (e.g. ECACC Accession No. 01090303 or 02082967)  
(ii) a cell which expresses CS5 and CS6 (e.g. ECACC Accession No. 01090305 or 02082968);



- (iii) a cell which expresses CS4 and CS6 (e.g. ECACC Accession No. 01090306 or 02082966);
- (iv) a cell which expresses CS2 and CS3 (e.g. ECACC Accession No. 01090304 or 02082964); and
- 5 (v) a cell which expresses CS1 and CS3 (e.g. PTL003, ECACC Accession No. 01090302 or 02082965).

As mentioned above, the cell deposited under ECACC Accession No. 01090302 (PTL003) has already been tested in two clinical trials and been shown to be safe and  
10 immunogenic.

The vaccine may be formulated using known techniques for formulating attenuated bacterial vaccines. The vaccine is advantageously presented for oral administration, for example as a dried stabilised powder for reconstitution in a suitable buffer prior  
15 to administration. Reconstitution is advantageously effected in a buffer at a suitable pH to ensure the viability of the bacteria. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered with each administration of the vaccine. Alternatively the vaccine is presented in a lyophilised encapsulated form.

20

The vaccine may be used in the vaccination of a mammalian host, particularly a human host. An infection caused by a microorganism, especially a pathogen, may therefore be prevented by administering an effective dose of a vaccine prepared according to the invention. The dosage employed may ultimately be at the discretion  
25 of the physician, but will be dependent on various factors including the size and weight of the host and the type of vaccine formulated. However, a dosage comprising the oral administration of from  $10^7$  to  $10^{11}$ , e.g. from  $10^8$  to  $10^{10}$ , bacteria per dose may be convenient for a 70 kg adult human host.

## **EXAMPLES**

The Examples described in this section serve to illustrate the invention.

### **Brief description of the drawings**

- 5     Figure 1: Map of suicide vector plasmid pDM4. u = unknown sequence, unknown length.

Figure 2: Map of improved suicide vector pJCB12.

- Figure 3: Diagram of method used to create specific gene deletion constructs by overlap extension PCR. Step 1 = PCR amplification of two DNA fragments. Step 2  
10    = overlap extension PCR using DNA products from reaction 1 and reaction 2 of step 1 and amplification of the overlap extension PCR product. R and S stand for restriction enzyme sites.

Figure 4: Diagram of method used to demonstrate correct integration of suicide vector in to targeted locus by linkage PCR.

- 15    Figure 5: Sequence of the ST gene and flanking regions of the plasmid in strain B showing the open reading frame of the structural gene and the position of all oligonucleotides described in the text and detailed in Table 1.

- Figure 6: Sequence of the EAST1 gene and flanking regions from IS1414 (Genbank # AF143819) showing the open reading frame of the structural gene and the position  
20    of all oligonucleotides described in the text and detailed in Table 1.

Figure 7: Sequence of the LT locus showing the position of the oligonucleotides used to create the deletion construct. The underlined ATG codon near the end of the sequence is the start codon of LTA.

- Figure 8: Sequence of the 3' flanking region of the ST-1 gene determined from  
25    strain E showing the position of oligonucleotides described in the text and detailed in Table 1. The open reading frame of the structural gene and the ATG and TAA start and stop codons are underlined.

Figure 9: Diagram of the individual stages involved in the attenuation of strain E.

Figure 10: Diagram of the individual stages involved in the attenuation of strain H.

Figure 11: Sequence of the *aroC* gene locus showing the position of the oligonucleotides used to create the deletion construct and of others mentioned in the text and described in Table 1.

Figure 12: Diagram of the individual stages involved in the attenuation of strain J.

Figure 13: Gel showing PCR amplified LT-B coding sequence from strain E. The left hand arrow indicates the LT-B PCR product and the right hand arrow indicates a 600bp marker.

Figure 14: Sequence of the LT-B gene cloned from strain E.

Figure 15: Diagram showing construction of plasmid for the expression of LT-B under control of the *nirB* promoter. The LT-B DNA was digested with BglII and NheI. The starting vector, pNCAT4, was digested with the same enzymes and the larger vector fragment was isolated. The LT-B DNA and vector fragment were ligated together. The KpnI and BglII sites in the final plasmid, pNLTB, may be used for cloning of the LT-B promoter.

Figure 16: Western blot showing the expression of LT-B in attenuated ETEC vaccine strain PTL003, in both the cytoplasm and periplasm.

Figure 17: Gel showing PCR amplified LTAB promoter sequence from strain E. The left hand arrow indicates a 200 bp marker and the right hand arrow indicates the LT promoter PCR product.

Figure 18: Sequence of LTAB promoter cloned from strain E.

Figure 19: Western blot showing the expression of LT-B in attenuated ETEC vaccine strain PTL003, under control of the native LT promoter. PLLTB-1,-2 and -3 are three independent colonies of PTL003 transformed with pLLTB. The three left hand arrows indicate background, the top right hand arrow indicates LTA and the bottom right hand arrow indicates LT-B.

Figure 20: Diagram of the “plasmid rescue” technique to obtain the sequence of the 3’ flanking region of ST of strain B. The technique is described in detail in Example 2. An ST cassette was made by PCR (primers 4764 and 4765) and cloned into plasmid pJCB12. The resulting pJCB12-ST plasmid was introduced into strain B by conjugation. Plasmid DNA from one of the transconjugants was isolated and digested with a restriction enzyme (*Bgl*II) which cuts at a site distant from the target gene. The 5’ and 3’ ends of the resulting cut plasmid were ligated together to close the plasmid. The plasmid was propagated in SY327 $\lambda$ *pir* and then sequenced with a primer (4792) which will only hybridise to the native ST gene (not to the ST gene of pJCB12-ST). The sequence obtained using this primer may be used to design further primers to extend the sequence.

**EXAMPLE 1: AN IMPROVED PROCESS FOR THE INTRODUCTION OF  
MULTIPLE GENTIC MUTATIONS INTO BACTERIAL VACCINE  
STRAINS**

5 This section describes the generation of a novel suicide vector plasmid, pJCB12, and its use in the generation of an optimised procedure for the introduction of mutations into chromosomal or plasmid encoded gene loci. It also describes many of the standard methods used in this and subsequent examples of the specification, at the end of the section. The sequence and purpose of the oligonucleotides used in PCR  
10 for construction or analysis of constructs are given in Table 1 at the end of the Examples.

**An improved process for the introduction of multiple genetic mutations into bacterial vaccine strains**

15 The generation of an attenuated ETEC strain from a wild-type strain requires mutation of a number of different genetic loci. These mutations are introduced sequentially, each using a process that requires several different steps. When a number of ETEC strains require attenuation, this amounts to a significant number of steps, each potentially with its associated difficulties. It is therefore vital that the  
20 process of introducing mutations is optimised fully.

Suicide vector plasmids such as pDM4 (20), pJCB12, pCVD442 (8) and others can be used to introduce defined genetic constructs into specific targets in the bacterial genome. Plasmid pJCB12 is a new, optimised suicide vector based on the previously  
25 constructed suicide vector pDM4. The defined genetic construct to be introduced into the bacterial genome may be a deletion mutation of a specific gene, or a more complex structure such as, for example, an insertion of a gene within another and expressed from a chosen promoter from within the construct. Generally, the

extremities of the constructs will consist of nucleotide sequences derived from the region of the genome to be targeted.

5 Suicide vectors pDM4 and pJCB12 possess a number of key components (see Figures 1 and 2):

10 An origin of replication which directs replication of the vector in some strains of bacteria but not in others, *oriR6K*. *oriR6K* is the origin of replication derived from the naturally occurring plasmid R6K. This origin requires the R6K *pir* gene for replication, which is absent from the suicide vectors. Three laboratory *E. coli* strains are available that carry the *pir* gene on their chromosome, which are SY327 $\lambda$ *pir*, SM10 $\lambda$ *pir*, and DH5 $\alpha$ *pir*. All three of these strains may be used to propagate pDM4, pJCB12 and their derivatives.

15 A transfer origin that directs conjugative transfer of the vector from one bacterial strain to another, *mobRP4*. *mobRP4* is the transfer origin from the naturally occurring plasmid RP4. This allows the conjugative transfer of pDM4 and pJCB12 and their derivatives to recipient bacterial strains. In order to function, *mobRP4* requires the genes encoding the RP4 transfer functions to be present in the donor bacterial cell. Laboratory *E. coli* strain SM10 $\lambda$ *pir* carries these genes on its  
20 chromosome, and so this strain can be used as a donor strain for pDM4, pJCB12 and their derivatives.

25 A gene encoding a product that is toxic to bacterial cells when the cells are grown under defined conditions, *sacB*. *sacB* codes for levansucrase which produces a product that is toxic to Gram-negative bacteria when grown on sucrose.

A selectable marker, *cat*. *cat* codes for chloramphenicol acetyltransferase and confers resistance to the antibacterial chloramphenicol.

5 A multiple cloning site (MCS), i.e. a site into which defined genetic constructs may be cloned for introduction into a recipient bacterial cell.

It is clear to us from our use that existing suicide vectors such as pDM4 and pCVD442 frequently does not target the correct locus, and screening of a large number of transconjugants or transformants is necessary to identify one that is  
10 correctly targeted (if a correctly targeted one can be identified at all). In addition, pCVD442 carries an ampicillin resistance determinant to allow selection of recombinants. However, selection on ampicillin is not as efficient as chloramphenicol and attempts to select for very small numbers of recombinants within a very large mixture of bacteria, such as when performing conjugation  
15 experiments, are often thwarted by “background growth”. “Background growth” can take the form of smears of bacteria within which identification of transconjugants is not possible, or small colonies, which upon further analysis are not transconjugants.

We believe that the problems of incorrect targeting are partly due to the relatively  
20 large size (6 – 7 kb) of these known suicide vectors which are themselves constructed from components of naturally occurring plasmids. The suicide vectors could therefore target naturally occurring plasmids within a bacterial strain that carry similar nucleotide sequences. In particular, transfer functions among conjugative plasmids are relatively conserved, and the *mob*RP4 transfer region of pDM4 and  
25 pCVD442 is approximately 2.5kb. In addition, in light of the problem of incorrect targeting we carried out sequencing. Our nucleotide sequence data obtained for the *sacB* region of pDM4 showed that it includes approximately 600bp of an *IS1*-like insertion sequence, an insertion sequence which is prevalent in the genomes of many bacteria. We deduced that this could be at least partly responsible for the incorrect  
30 targeting.

Suicide vector pJCB12 is a modified version of pDM4 in which much of the intergenic and non-functional DNA has been removed. Therefore, there is much less opportunity for incorrect targeting using this suicide vector. Whereas pDM4 is approximately 7 kb in size, pJCB12 is only 3 kb but retains all the key components.

5 In particular, the *mob*RP4 region of pJCB12 is merely 0.15 kb, and the IS1-like nucleotide sequences have been removed from the *sacB* region. These modifications are particularly advantageous when manipulating ETEC strains which generally harbour many plasmids that could act as undesirable targets of homologous recombination with components of the suicide vector. In addition, the smaller size of

10 pJCB12 allows easier *in vitro* manipulation and construction of derivatives because smaller DNA molecules ligate together and transform into *E. coli* hosts more efficiently, improving the chances of obtaining derivatives of the correct construction. The smaller size also allows greater efficiency when introducing the constructs into recipient bacteria by transformation rather than by conjugation.

15

Laboratory *E. coli* strain SM10 $\lambda$ *pir* can be used to transfer pJCB12 and its derivatives to recipient bacterial strains by conjugation because it has the *tra* functions from plasmid RP4 inserted into its chromosome. However, strain SM10 $\lambda$ *pir* shows relatively low transformation frequencies. For this reason, strain

20 DH5 $\alpha$ *pir* would normally be used for the construction of pJCB12 derivatives, and once derivatives of the correct construction have been identified these would be transferred to SM10 $\lambda$ *pir* for introduction to recipient strains by conjugation.

### Construction of suicide vector pJCB12

25 Suicide vector pJCB12 was constructed by several rounds of overlap extension PCR (31, Figure 3) using pDM4 plasmid DNA as template. Initially, four fragments were amplified from pDM4 by PCR using the high fidelity DNA polymerase, *Pfu* Turbo<sup>TM</sup>. These were the *ori*R6K fragment, amplified using oligonucleotides 4714 and 4715; the *mob*RP4 fragment amplified using oligonucleotides 4716 and 4717;

30 and the *cat* gene that was amplified in two parts using oligonucleotides 4718 with



4719 and 4720 with 4721. This was done in order to remove an *EcoRI* restriction enzyme site within the *cat* gene. The *oriR6K* fragment and the *mobRP4* were then joined in an overlap extension PCR reaction using oligonucleotides 4714 and 4717. Likewise, the *cat* fragments were joined using oligonucleotides 4718 and 4721.

5 These two resulting fragments were then joined in a final overlap extension PCR reaction using oligonucleotides 4717 and 4718. The resulting PCR product was ligated and transformed into SY327 $\lambda$ *pir* cells and transformants were selected on L-agar supplemented with chloramphenicol at 20  $\mu$ g/ml. Transformants harbouring plasmids of the correct size were obtained and one of these, called pDM4A7, was

10 chosen for further manipulation.

At this stage, clearly the *oriR6K* and *cat* components of the plasmid pDM4A7 are functional. However, in order to confirm that the *mobRP4* locus was functional plasmid pDM4A7 was transformed into strain SM10 $\lambda$ *pir*. These transformants were

15 picked onto L-agar supplemented with chloramphenicol at 15  $\mu$ g/ml and naladixic acid at 5  $\mu$ g/ml. This L-agar was cross-streaked with cells of strain SY327 $\lambda$ *pir*. While chloramphenicol selects those bacterial cells which harbour pDM4A7, nalidixic acid selects for SY327 $\lambda$ *pir*. After overnight incubation, many colonies grew where the strains were cross-streaked, but none grew elsewhere on the plate,

20 confirming that pDM4A7 is mobilisable from strain SM10 $\lambda$ *pir* and that the *mobRP4* locus is functional.

Plasmid pDM4A7 was then digested with *EcoRI*, treated with *Pfu* Turbo<sup>TM</sup> DNA polymerase and ligated in order to remove the *EcoRI* restriction enzyme site to

25 generate plasmid pDM4A7 $\Delta$ *EcoRI*. A short *HindIII* fragment from pDM4 which includes the multiple cloning site was then ligated into pDM4A7 $\Delta$ *EcoRI* digested with *HindIII*. The ligation reaction was transformed into SY327 $\lambda$ *pir* and transformants selected on L-agar supplemented with 20  $\mu$ g/ml chloramphenicol.

Oligonucleotide R6K-01 hybridises within the short *HindIII* fragment from pDM4 which includes the multiple cloning site. Therefore, transformants were screened by PCR using oligonucleotides R6K-01 and 4720 in order to identify those harbouring the desired plasmid construct. A number of such transformants were identified, and  
5 one of these, called pDM4A7ΔE, was chosen for further manipulation.

Plasmid pDM4A7ΔE carries three *EcoRI* sites very close together on the short *HindIII* fragment from pDM4 which includes the multiple cloning site. The two very short *EcoRI* fragments of pDM4A7ΔE were therefore removed by digestion with  
10 *EcoRI* followed by ligation. This resulted in a pDM4A7ΔE derivative that possess only one *EcoRI* site which was called pJCB10. The region of pJCB10 that includes oriR6K and the MCS was amplified using oligonucleotides 4715 and 4917 and nucleotide sequence determinations for part of this fragment were performed using oligonucleotide 4917. This presented us with the nucleotide sequence across the  
15 MCS which was previously unknown.

The *sacB* gene was then amplified using *Pfu* DNA polymerase and oligonucleotides 4722 and 4723. The 1.6 kb product was ligated with the plasmid vector pPCR-Script™ (Stratagene) and transformed into *E. coli* XL10 Gold™ cells (Stratagene).  
20 Transformants were obtained and the functionality of the *sacB* gene was confirmed by plating the clones onto L-agar and 5% sucrose agar. One construct gave good growth on L-agar, and none on 5% sucrose agar, and so was chosen as the source of the *sacB* gene. The *sacB* gene was then digested from this clone using the restriction enzyme *PstI*, sites for which were incorporated into oligonucleotides 4722 and 4723  
25 for this purpose, and ligated with pJCB10 also digested with *PstI*. Colonies were checked by PCR using oligonucleotides 4716 and 4766, yielding a product of the expected size (~1700bp). Again the functionality of the gene was confirmed by plating the clones onto L-agar and 5% sucrose agar. One construct grew on L-agar, but not on 5% sucrose agar. Sequencing of this construct using oligonucleotides 4716  
30 and 4766 respectively indicated the orientation of the *sacB* gene. This construct was called pJCB12.

### Principle of use of pJCB12

Once a defined genetic construct has been ligated into pJCB12 to give a pJCB12-derivative, the plasmid is transferred into a recipient strain such as an ETEC strain. This may be done according to methods well known in the art, either by conjugation  
5 from the pJCB12 host strain SM10 $\lambda$ *pir*, or by transformation of the purified pJCB12-derivative directly into the recipient strain.

Transconjugants or transformants are selected on bacteriological growth medium supplemented with the antibiotic chloramphenicol. Since the suicide vector pJCB12  
10 is unable to replicate in the absence of the *pir* gene, any transconjugants or transformants that grow will generally have resulted from fusion of the pJCB12-derivative with another replicon by homologous recombination. Using pDM4 and other larger suicide vectors will often result in incorrect targeting, and consequently mutants cannot be isolated in subsequent time-consuming steps.

15

Although targeting by pJCB12 is much improved over pDM4, incorrect targeting can still occur. Therefore, in order to optimise fully the defined mutation process, a novel approach was taken to screen transformants or transconjugants using PCR to identify those in which the pJCB12-derivative has targeted the desired region of the  
20 genome. For this, one oligonucleotide is designed which hybridises within the pJCB12 nucleotide sequences adjacent to the MCS where the defined genetic construct has been inserted. The other oligonucleotide is designed to hybridise to the region of the genome to be targeted, adjacent to but outside of the defined genetic construct. Transformants or transconjugants that are positive using this PCR will  
25 have the pJCB12-derivative targeted to the correct region of the genome (see Figure 4).

Once the correct recombinants have been identified, derivatives need to be isolated in which the pJCB12 vector has been lost. Such derivatives may be selected by  
30 supplementing the bacteriological growth medium with 5% sucrose. This sucrose

selection may be made more efficient using a modified L-medium in which the NaCl ingredient is absent and supplemented with 5% sucrose. Under these conditions the *sacB* gene of pJCB12 is toxic, and only derivatives where the *sacB* gene has been lost will grow. This event again occurs by homologous recombination and has a

5 number of outcomes. Firstly, a reversion event will result in the targeted region remaining as it was. Secondly, homologous recombination may result in the defined genetic construct being swapped with the targeted region resulting in the defined construct being incorporated at the target region. In addition, if the targeted region is part of a plasmid, such as many of the toxin genes of ETEC strains, then two

10 additional events may occur. These are, thirdly, an undefined spontaneous deletion event, resulting in the loss of a part of the targeted region which may extend beyond the boundaries of the defined genetic construct, and, fourthly, the loss of the whole plasmid, an event which may be termed "specific plasmid curing".

15 Testing of sucrose resistant derivatives by PCR can identify the desired recombinants. For this, oligonucleotides that hybridise at each end of the targeted region and outside of the defined genetic construct are used. If the PCR product is the same size as prior to introduction of the pJCB12-derivative construct, then a reversion event has occurred. If, for example the genetically defined construct is a

20 deletion mutation, then the PCR product should be smaller than previously and of a predictable size. Specific plasmid curing and undefined spontaneous deletion will normally result in no PCR product or non-specific products of unexpected size in this type of PCR reaction.

25 In summary, vector pJCB12 (or another similar vector of the invention) may be used in a method for producing a bacterial cell in which a target gene (e.g. a toxin gene such as ST, LT or EAST1 or a chromosomal gene such as an *omp* or *aro* gene) is deleted, inactivated or replaced, which method comprises transferring the vector into a bacterial cell containing the target gene and selecting for a cell in which the target

30 gene has been deleted, inactivated or replaced. The selection may be carried out using a multi-stage procedure along the following lines:

- Selecting for a colony of cells which contains the selectable marker. If the cell into which the vector is transferred is one that does not support replication of the vector from the origin of replication in the vector, selecting for such a colony of cells identifies cells in which the vector has become incorporated into a cellular replicon;
- 5
- Carrying out PCR to select for a cell in which the vector has correctly targeted to the target gene, wherein one of the primers used in the PCR hybridizes to vector sequence adjacent to the cloning site and the other hybridizes to a site in the cellular DNA adjacent to the target gene. A positive PCR indicates that the vector has
- 10 targeted to the target gene.
- Selecting for a cell from which vector sequence has been lost by growing the cell under conditions which make effective the gene encoding a product that is toxic to the cells when grown under defined conditions. Survival of a cell indicates that
- 15 vector sequence has been lost. Where the gene encoding the toxic product is *sacB*, the cell may be grown in medium supplemented with sucrose and from which NaCl is absent; the product of *sacB* is toxic when the cells are grown in this medium.
- Finally, PCR may be carried out using primers which hybridize at positions outside,
- 20 and adjacent to each end of, the target gene, wherein a PCR product smaller than the product obtained from a wild-type cell indicates a deletion mutation.

## Materials and Methods

- 25 **Bacterial strains used.** ETEC strains as described elsewhere in the specification and lab strains of E.coli:

Strain	Reference or Source
SY327 $\lambda$ pir	Miller and Mekalanos (56)
DH5 $\alpha$ pir	P. Barrow, Institute for Animal Health, Compton
30 SM10 $\lambda$ pir	Simon et al., 1983 (47)

- Bacteriological growth media.** ETEC strains were routinely grown in L-broth and on L-agar and incubated at 37°C overnight. L-broth consists of 10g/l peptone, 5g/l yeast extract and 5g/l of NaCl dissolved in 1 l of deionised water. L-agar is L-broth supplemented with 15g/l agar. Growth medium containing 5% sucrose was as described above, but without the 5g/l of NaCl. To optimise expression of CFAs ETEC strains were harvested from CFA-agar (1% casamino acids, 2% agar, 0.15% yeast extract, 0.005% MgSO<sub>4</sub>, 0.0005% MnCl<sub>2</sub>). Chloramphenicol was used at a concentration of 10 µg/ml, tetracycline at 15 µg/ml and streptomycin at 20 µg/ml.
- 10 **Bacterial Conjugations** were performed by mixing donor and recipient ETEC strains on L-agar and incubating at 37°C for 3 to 18 h. Bacterial growth was scraped off into L-broth and plated onto L-agar plates supplemented with chloramphenicol and another appropriate antibiotic to select ETEC strains (streptomycin for strain B, tetracycline for other ETEC strains) that had incorporated the pJCB12-derivative.
- 15 **Identification of correctly targeted recombinants.** Transconjugants or transformants obtained by growth on L-agar supplemented with chloramphenicol following introduction of pJCB12-derivative constructs were tested by PCR in order to identify those in which the desired genetic locus had been targeted. For this, one of the oligonucleotides hybridised within the pJCB12 nucleotide sequences adjacent to
- 20 the multiple cloning site (MCS) where the defined genetic construct had been inserted. The other oligonucleotide hybridised to the genome, adjacent to but outside of the defined genetic construct. In such a PCR, the generation of a fragment indicated that the binding sites for the respective oligonucleotides had become linked, which could occur only if the pJCB12-derivative had targeted the correct
- 25 region of the genome.
- Excision of pJCB12 from transconjugants by growth in the presence of 5% sucrose.** Transconjugants or transformants having the pJCB12-derivative targeted to the correct region of the genome were then streaked onto fresh L-agar supplemented with chloramphenicol and another appropriate antibiotic to select ETEC strains (see
- 30 above), and incubated at 37°C to allow colonies to grow. L-broth cultures inoculated from these fresh plates were then grown. Cells from these cultures were harvested, resuspended in 5% sucrose broth, and incubated overnight prior to plating serial

dilutions on 5% sucrose agar in order to select recombinants in which the pJCB12-derivative had excised. The inoculated sucrose agar plates were then incubated overnight and the resulting colonies tested by PCR using relevant oligonucleotides in order to identify mutants.

- 5    **DNA manipulations** were performed using standard procedures (25). Plasmid DNA was prepared using plasmid purification kits from QIAGEN (Crawley, UK) and DNA fragments were isolated from agarose gels using the QIAquick™ gel extraction kit from QIAGEN.
- 10   PCR reactions were performed routinely using *Taq* DNA polymerase (Life Technologies); when high fidelity PCR was required, such as in the construction of pJCB12, *Pfu* “Turbo” DNA polymerase (Stratagene) was used. Both DNA polymerases were used in accordance with the suppliers instructions. Routinely, the following PCR cycle was used:
- 15   Step 1; 95°C, 50 sec  
Step 2; 95°C, 10 sec  
Step 3; 55°C, 1 min  
Step 4; 72°C, 1 min  
Step 5; repeat steps 2 to 4 twenty-five times
- 20   Step 6; 72°C, 1 min
- For construction of pJCB12, a pDM4 plasmid DNA preparation was used as the template in PCR reactions. For routine PCR screening a pick from a bacterial colony was used as the template. For construction of toxin deletion mutations, plasmid DNA preparations extracted from suitable ETEC strains were used. Plasmid pJCB12
- 25   and DNA fragments incorporating deletion mutations were generated using a modification of overlap extension PCR (31). In the modified version, there is no overlap in the fragments amplified. Instead, the fragments flank the region to be

deleted, and the complementary sequences which allow joining of the two fragments are included in the 5'-ends of the relevant oligonucleotides; see Figure 3.

### SDS-PAGE

5 This was performed essentially as described by Laemmli (16) and was used for visualisation of expressed CFAs and for checking LPS profiles. For LPS profiles, cells were grown overnight in L-broth, harvested and resuspended in water so as to give a cell suspension with an A600 of 20/cm. The cell suspension was mixed with an equal volume of 50mM TrisHCl pH6.8, 2% (w/v) SDS, 10% (v/v) glycerol,  
10 0.25% (w/v) bromophenol blue, 2% (v/v) 2-mercaptoethanol and boiled for 5 minutes. Proteinase K was then added to a final concentration of 0.2 mg/ml and the samples incubated at 60°C for 1 h before loading 5 to 10 µl onto SDS-PAGE gels. For CFAs, bacteria were harvested from CFA-agar and resuspended in water so as to give a cell suspension with an A600 of 20/cm. The cell suspension was heated to  
15 65°C for 10 mins, the samples were then centrifuged to remove whole cells. An equal volume of 50mM TrisHCl pH6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 2% (v/v) 2-mercaptoethanol was then added. Volumes of 3 to 10 µl were then loaded onto 12% Tris-glycine SDS-PAGE gels (Invitrogen). CFAs were visualised by Coomassie blue staining (Sambrook et al 1989, ref 25)  
20 while LPS was visualised using a SilverXpress<sup>TM</sup> silver stain kit (Invitrogen).

### EXAMPLE 2: REMOVAL OF TOXIN GENES AND INTRODUCTION OF ATTENUATING MUTATIONS INTO STRAINS EXPRESSING CFA/I

#### 25 **Manipulation of Strain A to produce strain ACAM 2010**

Strain A (WS-1858B) expresses the colonisation factor antigen CFA/I and the heat-stable toxin ST. In order to generate an ST-negative derivative that continues to express the CFA/I colonisation factors the strain was first transformed with a plasmid conferring tetracycline resistance. This was to allow subsequent selection of the  
30 strain from conjugation mixtures. The plasmid conferring tetracycline resistance is a derivative of plasmid pACYC184 (40) in which the chloramphenicol resistance



determinant had been inactivated by deletion of a *BsmBI* restriction enzyme fragment using standard DNA manipulation procedures. This plasmid was termed “pACYC-Tc” and was introduced into strain A by electro-transformation.

- 5 The TetR derivative of Strain A was conjugated with SM10 $\lambda$ *pir* harbouring plasmid pJCB12-STI. This pJCB12 derivative contains a fragment of the STI gene amplified from strain B using oligonucleotides 4764 and 4765 cloned into the MCS. The sequence of this construct is given in Figure 5. Note that the STI fragment in this derivative is not a deletion; the aim is to target the locus with the markers encoded by
- 10 pJCB12 in order to allow selection for deletion events resulting in the correct outcome.

- Transconjugants were selected by plating the mixture on L-agar supplemented with tetracycline and chloramphenicol and the colonies obtained were confirmed as
- 15 transconjugants by PCR testing using oligonucleotides 4720 and 4721 which amplifies the chloramphenicol resistance determinant. Three transconjugants were identified and called A13, A14 and A18 respectively. Each transconjugant was grown on 5% sucrose medium and colonies obtained were screened for ST using oligonucleotides 4764 and 4765. One of the ST-negative derivatives identified was
- 20 called A18-34 and was tested by PCR for the presence of the *cfaB* gene using oligonucleotides BglIIFOR and BglmodREV, for the presence of the *cfaC* gene using oligonucleotides 4727 and 4728, and for the presence of the *cfaR* gene using oligonucleotides 4785 and 4786. All three of these PCR reactions were positive, confirming the presence of the relevant genes. Derivative strain A18-34 was then
- 25 grown on CFA-agar and processed for SDS-PAGE in order to visualise CFA/I expression. This confirmed that Strain A18-34 expressed CFA/I and, therefore, that an ST-negative, CFA/I expressing strain had been isolated.

- In the next step an ampicillin sensitive derivative of strain A18-34 was identified.
- 30 For this, strain A18-34 was grown in LB-broth through three passages. The resulting culture was then diluted and plated onto LB-agar. When small colonies were visible they were replica-plated onto LB-agar supplemented with ampicillin at 200 $\mu$ g/ml and then incubated to allow colonies to grow. One colony was identified that was absent

from the ampicillin supplemented replica plate and subsequently confirmed as being ampicillin sensitive. This strain was called A18-34 Ap<sup>S</sup>.

In the next step, a trimethoprim sensitive derivative of strain A18-34 Ap<sup>S</sup> was isolated. For this strain A18-34 Ap<sup>S</sup> was replica plated as described above but onto LB-agar and M9 minimal salts agar supplemented with 0.4% glucose and trimethoprim at 25 µg/ml. Two colonies were identified that had not grown on the trimethoprim supplemented replica plates, and one of these which was called A18-34 Ap<sup>S</sup> Tp<sup>S</sup> was chosen for further manipulations.

The EAST1 toxin gene was then deleted from the strain A18-34 Ap<sup>S</sup> Tp<sup>S</sup>. For this, conjugations were performed with SM10λ*pir* harbouring a pJCB12 plasmid derivative carrying a defined EAST1 deletion as the donor strain and strain A18-34 Ap<sup>S</sup> Tp<sup>S</sup> as the recipient strain. Transconjugants were selected on L-agar supplemented with tetracycline at 15 µg/ml and chloramphenicol at 10 µg/ml. One transconjugant was identified by PCR using oligonucleotides 4917 and 4778 in which the pJCB12-derivative was inserted at the correct location. This tetracycline and chloramphenicol resistant transconjugant was then grown in 5% sucrose medium in order to select recombinants in which the pJCB12-derivative had excised (as described in Materials and Methods). Colonies that grew on L-agar supplemented with 5% sucrose were then tested by PCR using oligonucleotides 4749 and 4752. Three isolates were identified that were negative in this PCR reaction indicating that the EAST1 locus had been lost.

Defined deletion mutations were then introduced into the *aroC*, *ompC* and *ompF* genes in order to further attenuate the A18-34 Ap<sup>S</sup> Tp<sup>S</sup> Δ*EAST1* derivative as described in other Examples. Initial attempts to introduce the Δ*aroC* mutation met with little success as was the case when constructing a strain J-derivative *aroC* deletion mutation (see below). Therefore, attempts were made to introduce the Δ*aroC*<sup>J</sup> mutation (see below for details of the mutation). Following transfer of pJCB12-Δ*aroC*<sup>J</sup> into the A18-34 Ap<sup>S</sup> Tp<sup>S</sup> Δ*EAST1* derivative a transconjugant was identified by PCR using oligonucleotides 4917 and 4742. This transconjugant was then grown in 5% sucrose medium in order to select recombinants in which the

- pJCB12-derivative had excised (as described in Materials and Methods). Colonies that grew on L-agar supplemented with 5% sucrose were then tested by PCR using oligonucleotides 47116 and 47117, and a derivative that carried the  $\Delta aroC^J$  defined deletion mutation was identified. Aromatic amino acid auxotrophy was
- 5 demonstrated in this A18-34 Ap<sup>S</sup> Tp<sup>S</sup>  $\Delta EAST1$   $\Delta aroC^J$  strain by streaking on minimal agar and minimal agar supplemented with “aro mix.” The strain grew only on the aro mix supplemented agar, whereas the parental strain A18-34 Ap<sup>S</sup> Tp<sup>S</sup>  $\Delta EAST1$  grew both in the presence and absence of “aro mix.”
- 10 Next the  $\Delta ompC$  defined deletion mutation was introduced into the A18-34 Ap<sup>S</sup> Tp<sup>S</sup>  $\Delta EAST1$   $\Delta aroC^J$ -derivative as described for strain H (Example 4) and a  $\Delta ompC$  deletion mutant was identified. Expression of CFA/I and LPS by this A18-34 Ap<sup>S</sup> Tp<sup>S</sup>  $\Delta EAST1$   $\Delta aroC^J$   $\Delta ompC$  strain was confirmed by SDS-PAGE.
- 15 Next the  $\Delta ompF$  defined deletion mutation was introduced into the A18-34 Ap<sup>S</sup> Tp<sup>S</sup>  $\Delta EAST1$   $\Delta aroC^J$   $\Delta ompC$  -derivative as described for strain H (Example 4) and a  $\Delta ompF$  deletion mutant was identified. Expression of CFA/I and LPS by this A18-34 Ap<sup>S</sup> Tp<sup>S</sup>  $\Delta EAST1$   $\Delta aroC^J$   $\Delta ompC$   $\Delta ompF$  strain was confirmed by SDS-PAGE.
- 20 Finally, the pACYC-Tc plasmid that was introduced into strain A to confer tetracycline resistance was specifically cured from the A18-34 Ap<sup>S</sup> Tp<sup>S</sup>  $\Delta EAST1$   $\Delta aroC^J$   $\Delta ompC$   $\Delta ompF$  strain. For this the plasmid pJCB12-pACYC<sub>ori</sub> described in Example 4 was introduced by electrotransformation. Transformants harbouring pJCB12-pACYC<sub>ori</sub> were selected by growth on L-agar supplemented with
- 25 chloramphenicol. One transformant was then grown in 5% sucrose medium in order to select derivatives from which the pJCB12-pACYC<sub>ori</sub> plasmid had been cured (as described in Materials and Methods). Colonies that grew on L-agar supplemented with 5% sucrose were then picked onto L-agar medium supplemented with chloramphenicol and onto L-agar medium supplemented with tetracyclin. This
- 30 identified a number of derivatives which were sensitive to both these antibiotics, confirming that the pJCB12-pACYC<sub>ori</sub> plasmid had indeed specifically cured the

strain of the pACYC-Tc marker plasmid and then itself been cured by growth in the presence of sucrose.

One of these antibiotic sensitive derivatives was chosen and tested by PCR for the presence of the *cfaA*, *cfaC*, and *cfaD* genes using oligonucleotide pairs 47104 with 47105, 4729 with 4730, and 4785 with 4786 respectively. In addition, the presence of the  $\Delta aroC^J$ ,  $\Delta ompC$  and  $\Delta ompF$  deletion mutations was confirmed using the oligonucleotide pairs 47116 with 47117, 4732 with 4743, and 4733 with TT1. Expression of CFA/I and LPS was confirmed in this strain using SDS-PAGE and the nucleotide sequence across the  $\Delta aroC^J$ ,  $\Delta ompC$  and  $\Delta ompF$  mutations was confirmed.

#### **Manipulation of Strain B to produce strain ACAM 2011**

Strain B (WS-4437A) expresses the colonisation factor antigen CFA/I and the heat-stable toxin ST. Initially, isolation of a spontaneous ST-deletion mutation was attempted by targeting ST using plasmid pJCB12-STI. This plasmid was introduced into Strain B by conjugation from SM10 $\lambda$ pir and transconjugants were obtained by growth on L-agar supplemented with chloramphenicol. After several attempts, a number of ST-negative mutants were identified, but these were all negative for CFA/I.

It was therefore decided to construct a defined STI deletion mutation specific for Strain B. The first requirement was to determine nucleotide sequence flanking the 3' end of the ST gene. The process used to do this is illustrated in Figure 20. Plasmid DNA was isolated from a transconjugant strain in which pJCB12-STI was targeted to the ST gene. Purified DNA was subjected to restriction endonuclease digestion with *Bgl*II which cuts the ETEC plasmid but not the ST gene or the pJCB12 construct.. Digested DNA was ligated and electrotransformed into SY327 $\lambda$ pir and transformants were selected on L-agar supplemented with chloramphenicol. This process is termed "plasmid rescue" and results in the re-isolation of the pJCB12 replicon incorporating a large fragment of DNA that includes the whole of the STI region and a large amount of flanking DNA. The sequence of the flanking DNA was obtained. Nucleotide sequence data obtained from this then allowed further

oligonucleotides (4797 and 4798) to be designed and used to determine additional nucleotide sequence further downstream of the STI gene in Strain B. Figure 5 shows the determined sequence and the location of all oligonucleotide binding sites.

- 5 Using the determined nucleotide sequence, an ST-deletion mutation was constructed. This was done by amplifying two fragments from the STI locus using oligonucleotides 47101 with 47114, and 47115 with 47100. The two resulting fragments were then joined by overlap extension PCR using oligonucleotides 47100 and 47101 and ligated into the MCS of pJCB12 using standard techniques.

10

- The construct was introduced into strain B by conjugation from SM10 $\lambda$ pir and chloramphenicol resistant transconjugants in which ST had been correctly targeted were identified by PCR using oligonucleotides 4917 and 4799. These transconjugants were grown in the presence of sucrose and colonies obtained were then screened using oligonucleotides 47100 and 47101 in order to identify ST deletion mutants. One derivative was found which was negative using these oligonucleotides, suggesting that a spontaneous deletion had resulted in loss of the entire ST locus. This derivative was positive by PCR using oligonucleotides 4727 with 4728 which amplify part of the *cfaC* gene. It was also positive using  
15 oligonucleotides BglIIFOR and BglmodREV which amplify the *cfaA* gene, and using  
20 oligonucleotides 4785 and 4786 which amplify the CFA/I regulator gene, *cfaD*. Functional assay for ST confirmed that this derivative was ST-negative, while SDS-PAGE confirmed that it continued to express CFA/I.

- 25 Defined deletions were then introduced into the *aroC*, *ompC* and *ompF* genes in order to attenuate the Strain B ST-negative derivative. Initial attempts to introduce the  $\Delta$ *aroC* deletion were unsuccessful. Therefore the  $\Delta$ *aroC*<sup>J</sup> deletion was used (see below for details of the mutation). Following transfer of the pJCB12 $\Delta$ *aroC*<sup>J</sup> deletion into Strain B ST-negative derivative, transconjugants were identified by PCR using  
30 oligonucleotides 4917 with 4742. A transconjugant was identified and then grown in 5% sucrose medium in order to select recombinants in which the pJCB12 derivative had been excised (as described in materials and methods). Colonies that grew on L-agar supplemented with 5% sucrose were then tested by PCR using oligonucleotides

47116 with 47117 and a derivative that carried the  $\Delta aroC^J$  deletion was identified. The CFA/I status of the exconjugant was checked by PCR using oligonucleotides 4727 with 4728 for *cfaC*, 4785 with 4786 for *cfaD* and BglIIFOR with BglIImodREV for *cfaA*.

5

The  $\Delta ompC$  defined deletion mutation was then introduced into the Strain B ST-negative  $\Delta aroC^J$  derivative as described for strain H (Example 4) and a  $\Delta ompC$  deletion mutant identified. The CFA/I status of the exconjugant was checked by PCR using oligonucleotides 4727 with 4728 for *cfaC*, 4785 with 4786 for *cfaD* and BglIIFOR with BglIImodREV for *cfaA*.

10

The  $\Delta ompF$  defined deletion mutation was introduced into the Strain B ST-negative  $\Delta aroC^J \Delta ompF \Delta ompC$  derivative as described for strain H (Example 4) and an *ompF* deletion mutant identified. The CFA/I status of the exconjugant was checked by PCR using oligonucleotides 4727 with 4728 for *cfaC*, 4785 with 4786 for *cfaD* and BglIIFOR with BglIImodREV for *cfaA*.

15

Finally the pStrep plasmid, that confers streptomycin resistance, was specifically cured from the Strain B ST-negative  $\Delta aroC^J \Delta ompF \Delta ompC$  derivative. For this a plasmid derivative of pJCB12 was constructed which incorporated the pStrep replication origin. This was done by shotgun cloning pStrep fragments generated by restriction endonuclease digestion with *SphI* into the *SphI* site of pJCB12, and transforming the DNA into XL-10 Gold ultracompetent *E. coli* cells (Stratagene). Transformants were plated on L-agar supplemented with chloramphenicol. Because pJCB12 is a suicide vector requiring special host strains that carry the *pir* gene, transformants that grew in the presence of chloramphenicol were assumed to be derivatives of pJCB12 which carried the pStrep replication origin. Plasmid DNA from four such transformants showed that in each case an *SphI* DNA fragment of approximately 2kb had been cloned into pJCB12. This pJCB12-derivative was called pJCB12-pStrep-ORI

25

30

The pJCB12-pStrep-ORI plasmid was introduced into the Strain B ST-negative  $\Delta aroC^J \Delta ompF \Delta ompC$  derivative by conjugation from strain SM10 $\lambda$ *pir*, and the

resulting transconjugants selected by growth on agar medium supplemented with chloramphenicol. Colonies which grew were used to inoculate broth supplemented with chloramphenicol and, following incubation to allow growth, dilutions of this culture were plated on agar supplemented with chloramphenicol. Colonies which  
5 grew on this medium were picked onto agar supplemented with streptomycin in order to identify those from which the pStrep plasmid had been lost. One of these streptomycin sensitive colonies was then grown in L-broth supplemented with 5% sucrose in order to select for a derivative from which the pJCB12-pStrep-ORI plasmid had been lost. Dilutions from this broth culture were plated on L-agar  
10 supplemented with 5% sucrose and after incubation some of the resulting colonies were picked onto agar supplemented with chloramphenicol in order to identify those from which the pJCB12-pStrep-ORI plasmid had been lost.

The CFA/I status of the exconjugant was again checked by PCR using  
15 oligonucleotides 4727 with 4728 for *cfaC*, 4785 with 4786 for *cfaD* and BglIIFOR with BglIImodREV for *cfaA*. CFA/I protein expression was then checked by SDS-PAGE and Western blotting. The LPS profile was also checked. In addition the  $\Delta aroC^J$ ,  $\Delta ompC$  and  $\Delta ompF$  mutations were confirmed by sequencing, and the aromatic amino acid dependence of the strain was also confirmed.

20

**EXAMPLE 3: A DERIVATIVE OF A VIRULENT WILD-TYPE ETEC  
STRAIN WHICH EXPRESSES CS5, CS6, LT, ST AND EAST1 WHEREIN  
THE LT, ST AND EAST1 GENES HAVE BEEN DELETED**

25

Strain E expresses CS5, CS6 and the toxins EAST1, ST and LT. In order to facilitate genetic manipulation plasmid pACYC-Tc (described in Example 2 above) was introduced into strain E by electro-transformation in order to confer tetracycline resistance.

30

The EAST1 toxin gene was deleted from Strain E first. This required the construction of a pJCB12 derivative which carries a defined EAST1 deletion mutation. Such a deletion mutation was generated by amplifying EAST1 fragments

from the ETEC strain H10407, using oligonucleotides 4749 with 4750, and 4751 with 4752, to generate two DNA fragments flanking the EAST1 gene. Figure 6 shows the sequence of the EAST1 locus derived from IS1414 (Genbank accession number AF143819) and all oligonucleotides used. These were then fused by an additional overlap extension PCR reaction using primers 4749 and 4752 and the resulting fragment was cloned into pJCB12 using the *SalI* and *SphI* restriction sites. Thus, a pJCB12 derivative had been constructed which incorporated this deletion mutation.

Strain SM10 $\lambda$ *pir* harbouring pJCB12- $\Delta$ EAST1 was conjugated with Strain E and transconjugants selected. Colonies that grew on L-agar supplemented with chloramphenicol and tetracycline were screened using the oligonucleotides 4917 and 4753 (equivalent to oligos 4 and 1 in Figure 4). Two transconjugants were obtained which were positive in both this PCR reaction indicating that the pJCB12- $\Delta$ EAST1 plasmid had correctly targeted the EAST1 gene.

These chloramphenicol resistant transconjugants were then grown in 5% sucrose medium in order to select recombinants in which the pJCB12-derivative had excised. Four exconjugants were obtained on the sucrose agar, and these were negative when tested for the presence of the EAST1 gene by PCR using oligonucleotides 4749 and 4752, indicating that the whole of the EAST1 region had been lost in this derivative.

The strain E EAST1-negative derivative was then tested by PCR using oligonucleotides 4738 with 4780 which are specific for the CS5 operon, and oligonucleotides 4740 with 4781 which are specific for the CS6 operon. These PCR reactions were positive for all four exconjugants confirming that they continued to harbour the CS5 and CS6 genes.

Next, an LT-deletion mutant of the Strain E EAST1-negative derivative was constructed. For this a pJCB12 derivative plasmid was constructed that carried a defined deletion of the LT-A gene in the LT locus. See Figure 7.



A defined LT-A deletion was constructed by PCR amplification using oligonucleotides 4772 with 4773, and 4774 with 4746, to generate two DNA fragments flanking the LT-A gene. These were then fused by an additional overlap extension PCR reaction and the resulting fragment was cloned into pJCB12 using the  
5 Sall and SphI restriction sites.

The pJCB12- $\Delta$ LT-A construct was introduced into the Strain E  $\Delta$ EAST1 derivative by conjugation from the pJCB12 host strain SM10 $\lambda$ pir and transconjugants were selected on L-agar supplemented with chloramphenicol and tetracycline. Resulting  
10 colonies were screened by PCR using oligonucleotides 4762 and R6K-01 which identified two transconjugants in which the LT locus had been correctly targeted.

These chloramphenicol resistant transconjugants were then grown in 5% sucrose medium in order to select recombinants in which the pJCB12-derivative had been  
15 excised. Colonies that grew on the sucrose agar were tested by PCR using oligonucleotides 4762 and 4746 in order to identify LT-A deletion mutants. Three derivatives were identified which were negative in this PCR reaction, suggesting that they had lost the entire LT locus. PCR reactions performed on these three LT-negative derivatives using oligonucleotides 4738 with 4780, and oligonucleotides  
20 4740 with 4781 confirmed the presence of the CS5 and CS6 genes respectively.

Next, the ST gene was deleted from this Strain E EAST1-negative LT-negative derivative. For this, plasmid pJCB12-STI (as described in Example 2) was transferred into Strain E from SM10 $\lambda$ pir and transconjugant selected on L-agar  
25 supplemented with chloramphenicol. A transconjugant was identified by PCR using oligonucleotides 4917 and 4794, and this transconjugant was then grown in 5% sucrose medium to select for derivatives from which the pJCB12-STI had been lost. Of 133 colonies screened, only 3 had lost STI, the others being revertants, and all of these 3 STI-negative derivatives were negative also for CS5 and CS6. These results  
30 suggested that the STI locus was present on the same plasmid as the CS5 and CS6 genes (as did Southern hybridization data) and that the STI locus was relatively stable, so that revertants or derivatives were obtained in which only specific plasmid curing had occurred.

It was therefore decided that a defined STI-deletion mutation specific for strain E was needed. In order to make this construct additional nucleotide sequence data was required for the region downstream of the STI gene in Strain E. Therefore, one of the pJCB12-ST transconjugants was used for sequence determinations at the STI  
5 locus using the plasmid rescue procedure described in Example 2 and illustrated in Figure 20. A pJCB12-derivative was obtained that incorporates a large fragment of DNA that includes the ST-I gene and a large amount of flanking DNA from Strain E. This plasmid preparation was used as template in nucleotide sequence determination reactions using oligonucleotide 4764 to determine sequence through the STI gene,  
10 and oligonucleotide 4792 to determine sequence downstream of the STI gene. The new sequence data allowed an additional oligonucleotide, 47106, to be designed which was used in further nucleotide sequence determinations. This additional new sequence data enabled oligonucleotides 47112, 47120 and 47121 to be designed for construction of the deletion cassette. The sequence of the ST-1 gene and flanking  
15 regions showing the binding sites of all oligonucleotides used is given in Figure 8.

As with other deletion mutations, two DNA fragments from the ST region were amplified by PCR using oligonucleotides 4764 with 47120, and 47121 with 47112,  
20 and the two fragments generated were fused by an additional overlap extension PCR reaction using oligonucleotides 4764 and 47112. The resulting fragment was cloned into pJCB12 using the *SacI* and *SalI* restriction endonuclease sites of pJCB12 and those incorporated into oligonucleotides 4764 and 47112 respectively.

25 The resulting recombinant plasmid pJCB12- $\Delta$ STI<sup>E</sup> was introduced into the Strain E EAST1-negative LT-negative derivative from strain SM10 $\lambda$ *pir*. The resulting chloramphenicol and tetracycline resistant transconjugants were screened by PCR using oligonucleotides 4917 with 4794, and R6K-01 with 47113. Two  
30 transconjugants were identified that were positive, indicating that the STI gene had been correctly targeted.

These chloramphenicol resistant transconjugants were then grown in medium supplemented with sucrose, followed by plating on 5% sucrose agar to obtain

excision of the pJCB12 derivative. The colonies that grew on sucrose agar were screened using oligonucleotides 4764 with 47112 and a number were identified that were negative, and three colonies were identified that showed the presence of the STI deletion mutation. PCR reactions performed on the STI deletion mutants using  
5 oligonucleotides 4738 with 4739 to detect CS5, 4740 and 4741 to detect CS6 and 4783 with 4784 to detect the CS5 regulator gene were all positive, indicating that these genes had been retained in these defined STI-deletion mutants. Additional attenuating mutations in *aroC*, *ompC* and *ompF* were introduced as described (ref 32 and Example 4). See Figure 9.

10

**EXAMPLE 4: REMOVAL OF TOXIN GENES AND INTRODUCTION OF  
ATTENUATING MUTATIONS INTO STRAINS EXPRESSING CS2/CS3  
(STRAIN H) AND CS4/CS6 (STRAIN J)**

15 **Strain H Manipulation**

Strain H expresses CS2 and CS3 and the toxins EAST and ST. It also has the genes for LT, but LT protein has not been detected by *in vitro* assays. In order to facilitate genetic manipulation the plasmid pACYC-Tc (described in Example 2) was introduced into Strain H by electro-transformation in order to confer tetracycline  
20 resistance.

The EAST1 toxin gene was the first to be deleted from Strain H. Strain SM10 $\lambda$ *pir* containing the pJCB12- $\Delta$ EAST1 plasmid described in Example 3 was conjugated with Strain H and transconjugants selected on L-agar containing chloramphenicol and tetracycline. Transconjugant colonies in which the EAST1 gene had been  
25 correctly targeted were identified by PCR using the oligonucleotides 4917 and 4753. Transconjugants were then processed in order to select derivatives in which the pJCB12-derivative had excised (described above). Colonies that grew on 5% sucrose agar were tested by PCR using the oligonucleotides 4775 and 4777 and a number  
30 were identified that were negative by PCR indicating that the whole of the EAST1 region had been lost. These Strain H EAST1-negative derivatives were then tested for the presence of a transcriptional activator for colonization factors, *rns*, by PCR using the oligonucleotides RNS-03 and RNS-04. Two of the Strain H EAST1-

negative mutants were positive for *rns*. Further testing by PCR for CS2 using oligonucleotides 4712 and 4779, and CS3 using oligonucleotides CS3-02 and CS3-03 indicated that the mutants were both CS2 and CS3 positive. Testing by PCR for the LT locus using oligonucleotides LT-04 and LT-05, and for the STI locus using  
5 oligonucleotides EST-01 and 4765 showed that the mutants were negative for these toxins, indicating that the ST and LT loci had been lost concomitantly with EAST1. The expression of CS2 and CS3 in the Strain H toxin-negative mutants was confirmed by SDS-PAGE.

10 Defined deletion mutations were then introduced into the *aroC*, *ompC*, and *ompF* genes in order to further attenuate the Strain H toxin-negative derivative using a method similar to that described previously (32). However, this earlier description for introducing these mutations used the suicide vector pCVD442 described in Example 1. This suicide vector codes for ampicillin resistance, which is not optimal  
15 for selecting infrequent transconjugant from a large mixed bacterial population. In addition, at this stage, the Strain H toxin-negative derivative continues to express its own ampicillin resistance, making pCVD442 useless with this strain. Therefore, the  $\Delta$ *aroC*,  $\Delta$ *ompC* and  $\Delta$ *ompF* deletion mutations previously cloned into pCVD442 (32) were sub-cloned into pJCB12. For this, the  $\Delta$ *aroC* mutation was sub-cloned using  
20 the restriction endonuclease sites *Xba*I and *Sac*I,  $\Delta$ *ompC* used *Sac*I and *Sal*I sites, and  $\Delta$ *ompF* used *Sac*I and *Sph*I sites. The pJCB12-derivatives were then transformed into strain SM10 $\lambda$ *pir*.

The defined  $\Delta$ *aroC* mutation was the first to be introduced into the Strain H toxin-  
25 derivative from strain SM10 $\lambda$ *pir*. Transconjugant colonies that grew on L-agar supplemented with chloramphenicol and tetracycline were screened by PCR using the oligonucleotides 4917 and 4742 in order to identify those in which the *aroC* locus had been correctly targeted. These transconjugant colonies were then streaked onto fresh L-agar supplemented with chloramphenicol and tetracycline. Following  
30 incubation, the colonies that grew were tested for the presence *rns* by PCR using the oligonucleotide primers RNS-03 and RNS-04 and for CS2 using primers 4712 and 4779. The reactions confirmed that the transconjugants were positive for both these loci. Transconjugants were then processed in order to select derivatives in which the

pJCB12-derivative had excised (as described in previous Examples). Colonies that grew on 5% sucrose agar were tested by PCR using the oligonucleotide primers 4731 and TT20 in order to identify derivatives that harboured the defined  $\Delta aroC$  deletion mutation. One defined  $\Delta aroC$  deletion mutant was identified and again checked by  
5 PCR to confirm the presence of *rns* and CS2, as described above. Expression of CS2 and CS3 by this defined  $\Delta aroC$  deletion mutant was confirmed using SDS-PAGE, and its LPS was checked using SDS-PAGE.

Next the *ompC* defined deletion mutation was introduced into this Strain H toxin-negative  $\Delta aroC$  derivative by conjugation from strain SM10 $\lambda$ pir harbouring pJCB12- $\Delta ompC$ . Transconjugant colonies that grew on L-agar supplemented with chloramphenicol and tetracycline were screened by PCR using the oligonucleotides 4917 and 4743 in order to identify those in which the *ompC* locus had been correctly targeted. Transconjugants were then processed in order to select derivatives in which  
15 the pJCB12-derivative had excised (as described above). Colonies that grew on 5% sucrose agar were tested by PCR using the oligonucleotide primers 4732 and 4743 in order to identify derivatives that harboured the defined  $\Delta ompC$  deletion mutation.

The defined *ompF* mutation was then introduced into this Strain H toxin-negative  $\Delta aroC$   $\Delta ompC$  derivative in a similar fashion to that described for the *ompC* mutation above, except that transconjugant colonies were screened by PCR using the oligonucleotides R6K-01 and 4733 in order to identify those in which the *ompF* locus had been correctly targeted. Colonies that grew on 5% sucrose agar were tested by PCR using the oligonucleotides 4733 and TT1 in order to identify  
25 derivatives that harboured the defined  $\Delta ompF$  deletion mutation.

All of the defined deletion mutations of the Strain H toxin-negative  $\Delta aroC$   $\Delta ompC$   $\Delta ompF$  derivative were then checked by PCR for comparison with the wild-type Strain H. For this the oligonucleotides used for *aroC* were 4731 and TT20, for *ompC*  
30 were 4732 and 4743, and for *ompF* were 4733 and TT1. The PCR products generated by these reactions were used for nucleotide sequence determinations across the deletion mutations. The oligonucleotides used for the nucleotide sequence determination reactions were TT35, TT38 and TT33 for *aroC*, *ompC* and *ompF*

respectively. All the deletion mutations had the expected nucleotide sequence. The mutant was also checked by PCR for the presence of *rns* and CS2, and for the absence of the toxin loci, as described above. The presence of the CS3 locus was also confirmed by PCR using the oligonucleotides CS3-03 and CS3-06. Expression  
5 of CS2 and CS3 was confirmed using SDS-PAGE, and the LPS was checked using SDS-PAGE.

The ampicillin resistance determinant was then removed from the Strain H toxin-negative  $\Delta aroC$ ,  $\Delta ompC$ ,  $\Delta ompF$  derivative. For this the derivative strain was  
10 grown through three passages in L-broth and then dilutions plated on L-agar in order to obtain well-separated colonies. The colonies were then replica plated onto L-agar supplemented with ampicillin at 100  $\mu$ g/ml. After incubation to allow the colonies to re-grow, the L-agar plates were examined in order to identify any colonies present on the L-agar that did not grow on the ampicillin supplemented L-agar. One such  
15 colony was identified and used in further experiments.

Finally, the pACYC-Tc plasmid that was introduced into Strain H to confer chloramphenicol resistance was specifically cured from the Strain H toxin-negative  $\Delta aroC$ ,  $\Delta ompC$ ,  $\Delta ompF$  derivative. This required the construction of the plasmid  
20 pJCB12-pACYC*ori*. For this the pACYC-Tc origin of replication was amplified by PCR using the oligonucleotides 4760 and 4761 and cloned into pJCB12 using the restriction endonuclease sites *SacI* and *SalI*. Strain SM10 $\lambda$ pir containing pJCB12-pACYC*ori* was conjugated with the Strain H toxin-negative-  $\Delta aroC$ ,  $\Delta ompC$ ,  $\Delta ompF$  derivative, and transconjugants were selected on L-agar containing chloramphenicol.  
25 It was hoped that by introducing a second plasmid carrying the same pACYC184 replication origin and selecting for its maintenance, the first pACYC-Tc plasmid would be rendered unstable and in the absence of any selection for its maintenance would be more frequently lost spontaneously. Chloramphenicol resistant transconjugant colonies harbouring the pJCB12-pACYC*ori* plasmid derivative were  
30 picked onto L-agar supplemented with tetracycline and one of these colonies was found to be tetracycline sensitive, indicating that the pACYC-Tc plasmid had been lost. This tetracycline sensitive, chloramphenicol resistant colony was then grown on 5% sucrose medium in order to select for derivatives from which the pJCB12-

pACYC*Cori* had been lost spontaneously. Colonies that grew on 5% sucrose agar were picked onto L-agar supplemented with chloramphenicol to confirm that the pJCB12- pACYC*Cori* plasmid had indeed been lost.

- 5 These manipulations are shown schematically in Figure 10.

### Strain J Manipulation

Strain J expresses CS4 and CS6 and the toxins EAST and ST. It also has the genes for LT, but LT protein has not been detected by *in vitro* assays. In order to facilitate  
10 genetic manipulation the plasmid pACYC-Tc (described in Example 2) was introduced into strain J by electro-transformation thus conferring tetracycline resistance.

The EAST1 toxin gene was deleted from strain J first. Strain SM10 $\lambda$ *pir* containing  
15 pJCB12- $\Delta$ EAST1 was conjugated with Strain J and transconjugants selected on L-agar containing chloramphenicol and tetracycline. Colonies that grew were screened by PCR using the oligonucleotides 4917 and 4753 in order to identify those in which the EAST1 gene had been correctly targeted. These transconjugant colonies were then streaked onto fresh L-agar supplemented with chloramphenicol and tetracycline  
20 and incubated to allow colonies to grow. These colonies were tested by PCR to confirm the continued presence of the CS4 operon using the oligonucleotides 4768 and 4769, and for the CS6 operon using oligonucleotide 4740 and 4781.

One transconjugant which was positive for both these PCR reactions was processed  
25 in order to select derivatives in which the pJCB12-derivative plasmid had excised (described above). Colonies that grew on 5% sucrose agar were tested by PCR using the oligonucleotides 4749 and 4752 to identify EAST1 mutants. All colonies tested were negative by this PCR reaction indicating that the whole of the EAST1 region had been lost in these derivatives. Further PCR reactions were performed to test for  
30 the continued presence of the CS4 and CS6 genes as described above, and expression of CS6 was confirmed by SDS-PAGE.

PCR reactions using oligonucleotides LT-04 and LT-05 amplified a product of the expected size for the LT locus in the Strain J EAST1-negative derivative. Nucleotide sequence determination reactions using these same oligonucleotides showed that this fragment generated by PCR was indeed of the LT locus. Therefore, this locus was  
5 targeted using the pJCB12- $\Delta$ LT-A construct in the same way as described in Example 3. LT-negative derivatives were identified in which the whole of the LT locus had been deleted. Further PCR reactions to test for the presence of CS4 using oligonucleotides 4768 and 4769, and CS6 using the oligonucleotides 4740 and 4781 confirmed the continued presence of these loci in the LT-negative derivatives. The  
10 expression of CS6 by the Strain J EAST1-negative LT-negative derivatives was confirmed by SDS-PAGE.

PCR reactions performed using the oligonucleotides ST-01 and ST-02 indicated that the ST present in Strain J was that encoded by the transposon Tn/681 (41). The  
15 Strain J EAST1-negative LT-negative derivatives were tested by PCR using the oligonucleotides ST-01 and ST-02, and were shown to be ST-negative.

The *aroC* locus of the Strain J toxin-negative derivative was targeted using pJCB12- $\Delta$ *aroC* as described above for Strain H. Correctly targeted transconjugants were  
20 identified. Unusually, however, following growth on 5% sucrose medium all derivatives generated in a large number of experiments were identified as revertants. Therefore a new pJCB12- $\Delta$ *aroC* construct was made that incorporated a smaller deletion in the *aroC* locus. This was constructed by PCR amplification using oligonucleotides 47116 with 47118, and 47119 with 47117, to generate two DNA  
25 fragments flanking that region of the *aroC* gene to be deleted. These were then fused by an additional overlap extension PCR reaction using oligonucleotides 47116 with 47117, and the resulting fragment was cloned into pJCB12 using the *Xba*I and *Sac*I restriction endonuclease sites. This construct was called pJCB12- $\Delta$ *aroC*<sup>J</sup> and was electrotransformed into SM10 $\lambda$ *pir*. The sequence of the *aroC* gene and the binding  
30 sites of the oligonucleotides used to construct this novel deletion construct are shown in Figure 11.



While pJCB12- $\Delta aroC^J$  was undergoing construction, the *ompC* defined deletion mutation was introduced into the Strain J toxin-negative derivative using the pJCB12- $\Delta ompC$  construct and procedure described for Strain H. One *ompC* defined deletion mutant was identified by PCR using oligonucleotides 4732 and 4743.

5

The defined *aroC* deletion mutation was then introduced into this Strain J toxin-negative  $\Delta ompC$  mutant using the new pJCB12- $\Delta aroC^J$  construct by conjugation from strain SM10 $\lambda pir$ . Colonies that grew on L-agar supplemented with chloramphenicol and tetracycline were screened by PCR using the oligonucleotides 4917 and 4742 in order to identify those in which the *aroC* gene had been correctly targeted. Transconjugants were then processed in order to select derivatives in which the pJCB12-derivative plasmid had excised (described above). Colonies that grew on 5% sucrose agar were tested by PCR using the oligonucleotides 4731 and TT20 in order to identify those with the incorporated defined *aroC^J* deletion mutation.

15

The defined *ompF* deletion mutation was then incorporated into an identified Strain J toxin-negative  $\Delta ompC \Delta aroC$  derivative. This was done exactly as described above for strain H, and one *ompF* deletion mutant was identified.

20 The tetracycline resistant plasmid pACYC-Tc was then specifically cured from the toxin-negative  $\Delta ompC \Delta aroC \Delta ompF$  derivative of Strain J using pJCB12-pACYC*ori* and then this plasmid itself was removed, again as described above for Strain H.

25 Finally, PCR reactions were performed to confirm the presence of CS4 using oligonucleotides 4768 with 4769, and CS6 using the oligonucleotides 4740 with 4781. Similarly the presence of the CFA/IV regulatory gene was confirmed using oligonucleotides 4785 and 4786. The nucleotide sequences of the defined *aroC*, *ompC* and *ompF* deletion mutations were determined as described for Strain H, and  
30 were as expected. The absence of EAST1, LT and ST loci were again confirmed by PCR as described above, expression of CS6 was confirmed by SDS-PAGE, and the LPS also was checked using SDS-PAGE.

A diagram showing all of the steps involved in these manipulations is shown in Figure 12.

#### **EXAMPLE 5: INCREASING THE STABILITY OF MANIPULATED CFA PLASMIDS**

This Example describes as an illustration one system for stabilising a CFA plasmid. The *parDE* locus was used as the stabilizing moiety.

10 In order to obtain a derivative of an ETEC strain that does not code for ST but continues to express its CFA genes, a pJCB12-ΔSTI derivative is constructed where the *parDE* locus is flanked by regions homologous to the regions flanking the ST gene in the plasmid to be targeted. Introduction of this plasmid into a recipient strain and selection on chloramphenicol will now result in transconjugants in which both  
15 loci are present in the CFA plasmid. The functional *parDE* locus will now ensure that this plasmid is maintained in these cells. Growth on sucrose to identify derivatives from which the pJCB12 component has been lost will now strongly select for cells containing a recombinant plasmid in which the desired cross over (ie the replacement of the ST structural gene with the *parDE* locus) has taken place. Cells  
20 in which a reversion event has occurred or from which the entire plasmid has been lost will be killed by the action of the toxin. Incorporation of the *parDE* locus will also function to stabilise the inheritance of the CFA plasmid during subsequent rounds of mutation, for example the introduction of additional attenuating mutations such as in the *aroC*, *ompC* and *ompF* genes.

25 Oligonucleotides 4789 and 4790 are used to amplify the *parDE* locus from plasmid RP4. The purified PCR product is then cloned into pJCB12-ΔSTI using the *Xho*I restriction enzyme sites to give plasmid pJCB12-ΔSTI::*parDE*. This plasmid is now introduced into a recipient strain by conjugation from SM10λpir or electro-  
30 transformation with purified plasmid DNA and the required intermediates and derivatives isolated by methods described in detail in the previous Examples.

**EXAMPLE 6: EXPRESSION OF LT-B IN AN ATTENUATED ETEC STRAIN, PTL003, TO INDUCE A PROTECTIVE IMMUNE RESPONSE AGAINST LT**

5     **Aim**

The aim of this work was to express the B-subunit of *Escherichia coli* heat labile toxin in a vaccine strain of ETEC. The LTB gene was derived from strain WS2773-E (NAMRU3, Cairo, Egypt) but it could be derived from any LTB-encoding strain. Similarly the approach could be extended to include mutants of LT-A, LT-B fused to  
10     other proteins (eg ST) or to expression of CT-B or derivatives thereof. Initial plasmid constructs were designed to demonstrate that LT-B could be expressed in an ETEC vaccine strain in the absence of LT-A and could be correctly exported and assembled. Subsequent constructs used the native LT promoter to drive expression. Ultimately, a similar construct could be inserted into the chromosome of ETEC to  
15     create a stable strain without the need for antibiotic selection.

**Methods**

**Section 1 – PCR amplification of the LTB CDS (protein coding sequence)**

20     **Primers**

Genbank sequences were used to design appropriate PCR primers (Table 1). The forward primer (Bfor) was designed to amplify from the start codon of the LTB gene and was based on Genbank sequence M17874 (17). To facilitate cloning into expression vectors, a BglIII restriction site was included starting 8 bases 5' prime of  
25     the ATG start codon. The reverse primer (Brev) was designed to amplify from 200 bases downstream of the stop codon, such that any transcription terminators would be included in the PCR product, and was based on Genbank sequence AF190919 (26). An NheI restriction site was included in the primer to facilitate cloning into expression vectors.

**Template**

Plasmid DNA was isolated from Strain WS2773-E (NAMRU3, Cairo, Egypt) for use as a template.

**5 Reaction**

10	Reaction mixture	2 µl plasmid DNA from WS2773-E (approx 50 ng/µl)
		10 µl 10X Pfu DNA Polymerase buffer (Stratagene™)
		0.8 µl dNTPs (25 mM of each dNTP)
		0.5 µl primer BFOR (258 ng/µl)
		0.5 µl primer BREV (227 ng/µl)
		84 µl H <sub>2</sub> O
		2 µl PfuTurbo™ DNA Polymerase(2.5U/µl)

15	Program	1 cycle	94°C x 1 min
		30 cycles	94°C x 1 min
			56°C x 1 min
			72°C x 1 min
		1 cycle	72°C x 10 min
		Hold	4°C

20

**Results**

A 600 bp PCR product was synthesized and isolated from a 1% agarose gel using a QIAquick™ gel extraction kit (Qiagen) according to the manufacturer's instructions (Figure 13).

## Section 2 – Cloning of the LTB PCR product

### **Cloning into pPCR-Script Amp SK+**

Gel isolated PCR product was ligated into pPCR-Script Amp SK+<sup>TM</sup> (Stratagene) according to the instructions in the manufacturer's instruction manual (#211188). 2 µl of ligation mix was used to transform *E. coli* XL10-Gold<sup>TM</sup> Supercompetent cells (Stratagene #230350) and correct constructs were identified by digestion of purified plasmid DNA with PvuII. A correct construct was designated pPCRLTB. The LTB gene was fully sequenced (Fig 14) and was compared to Genbank sequence M17874 (17). Four base changes were found which resulted in two amino acid changes. The PCR was repeated and a second clone was sequenced and gave identical results, showing that the LT-B sequence in strain WS2773-E differs from the database sequence.

### **Cloning into an inducible expression vector**

The LTB gene was transferred into expression vector pNCAT4 (Fig 15). pNCAT4 is an expression vector designed for use in *Salmonellas typhi* and *typhimurium*. The vector was originally derived from pTETnir15 (3) but had been modified by replacement of the TetC gene by the *H. pylori* catalase gene and replacement of the *bla* ampicillin resistance gene by a kanamycin resistance gene. In this plasmid the catalase gene is under the control of the *nirB* promoter which is upregulated in anaerobic conditions. For the purpose of the present invention many alternative expression plasmids which are well known in the art could be substituted for pNCAT4 or pTETnir15.

25

Plasmid pPCRLTB was digested with restriction enzymes BglII and NheI and a 600 bp fragment containing the LTB gene was isolated from a 1% agarose gel using a QIAquick<sup>TM</sup> gel extraction kit (Qiagen) according to the manufacturer's instructions. Plasmid pNCAT4 was digested with restriction enzymes BglII and NheI and a 2.6 kb

vector fragment was isolated from a 1% agarose gel using a QIAquick™ gel extraction kit (Qiagen) according to the manufacturer's instructions.

5 The vector was ligated to the LTB gene (25) and the ligation mixture was used to transform *E. coli* XL10-Gold™ supercompetent cells (Stratagene #230350). Correct clones were identified by restriction enzyme analysis and one (designated pNLTB) was used to transform electrocompetent ETEC-PTL003.

### Section 3 - Expression and localization of LTB in ETEC-PTL003

10

#### **Sample preparation**

Cultures of PTL003 and PTL003-pNLTB were grown overnight in LB medium with appropriate antibiotics. Samples (1 ml) were diluted in 100 ml medium and grown with shaking for ~3 h. Flasks were transferred to a static incubator and growth was  
15 continued for a further 1.5 h.

Bacteria were fractionated into periplasmic and spheroplast components by the following method: Cells were harvested by centrifugation for 5 min at 10 400 x g. The cell pellet was resuspended in 5 ml of 200 mM Tris HCl, pH8.0 and was  
20 transferred to a 50 ml glass beaker containing a magnetic flea. To this was added 5 ml of TES buffer (200 mM Tris HCl, pH 8.0, 1 mM Na<sub>2</sub>EDTA, 1 M sucrose). Stirring was set in motion and a timer was started. At t=90 s, 1 mg of lysozyme (10 mg/ml solution) was added, and at t=135s, 10 ml of water was added; the mixture was incubated at room temperature for a further 30 min. Spheroplast formation was  
25 checked with a light microscope and, if complete, the preparation was centrifuged for 20 min at 47 500 x g at 20°C. The supernatant containing the periplasmic proteins was harvested and concentrated ~ two-fold in a Centricon Macrosep™ spin-concentrator (3 000 NMWL, Millipore). The pellet, containing spheroplast proteins (cytoplasmic and membrane-bound), was resuspended in PBS. Samples of the cell  
30 fractions were mixed with equal quantities of Tris-Glycine SDS-PAGE sample

buffer (Invitrogen LC267) containing 0.1 M dithiothreitol. A portion of the periplasmic sample was heated at 95°C for 5 min, the remainder was kept at room temperature.

## 5 Sample analysis

Samples were separated by electrophoresis on 18% Tris-Glycine Gels (Invitrogen EC6505) and transferred onto nitrocellulose membranes according to the instructions supplied with the Xcell II Blot Module<sup>TM</sup> (Invitrogen EI9051). Blots were incubated as follows:

10

Block 1 hour in PBS, 0.05% Tween 20<sup>TM</sup> containing 5% dried milk (Marvel<sup>TM</sup>) with gentle rocking.

15

Primary antibody 1 hour with rabbit anti-cholera toxin antibody (Sigma C3062) diluted 1:10 000 in PBS, 0.05% Tween 20<sup>TM</sup> containing 1% Marvel and a 1:2 000 dilution of an *E. coli* extract (Promega #S3761). The antibody/extract mixture was pre-incubated for 1 hour before use to reduce non-specific binding to *E. coli* proteins.

Wash Three 5 min washes in PBS, Tween 20<sup>TM</sup>, 1% Marvel<sup>TM</sup>.

20

Secondary antibody 1 hour with horseradish peroxidase conjugated anti-rabbit antibody (Sigma A4914) diluted 1:10 000 in PBS, 0.05% Tween 20<sup>TM</sup>, 1% Marvel<sup>TM</sup>.

Wash Three 5 min washes in PBS, Tween 20<sup>TM</sup>, 1% Marvel<sup>TM</sup>.

25

Two 5 min washes in PBS, Tween 20<sup>TM</sup>

Two 5 min washes in PBS

Develop Blots were developed using a SuperSignal West Pico Chemiluminescent Substrate<sup>TM</sup> kit (Pierce).

The results of the Western blots are presented in Figure 16. LTB monomers were detected in both spheroplast and periplasmic boiled fractions. In the periplasmic samples kept at room temperature LTB pentamers were detected indicating that the LTB could be transported across the cell membrane and assembled in the normal way.

#### **Section 4 – PCR amplification of the LTB promoter**

##### **Primers**

10 PCR primers were designed based on preliminary sequence data of the upstream region of the LTA gene of WS2773-E (Figure 7). The forward primer (Pfor) annealed ~ 200 bp upstream of the LTA gene. A *KpnI* restriction site was included in the primer to facilitate cloning into expression vectors. The reverse primer (Prev) annealed just upstream of the start codon of the LTA gene and was designed to  
15 introduce a *BglII* restriction site to allow correct positioning of the promoter fragment with respect to the LTB gene.

##### **Template**

Plasmid DNA was isolated from Strain WS2773-E (NAMRU3, Cairo, Egypt) for use  
20 as a template.

##### **Reaction**

Reaction conditions were as described for the LTB gene in Section 1.



## Results

A 200 bp PCR product was synthesized and isolated from a 1% agarose gel using a QIAquick<sup>TM</sup> gel extraction kit (Qiagen) according to the manufacturer's instructions. (Figure 17)

5

### Section 5 – Cloning of the LT promoter

#### Cloning into pPCR-Script Amp SK+

Gel isolated PCR product was ligated into pPCR-Script Amp SK<sup>TM</sup>+ (Stratagene) according to the instructions in the manufacturer's instruction manual (#211188). 2  $\mu$ l of ligation mix was used to transform *E. coli* XL10-Gold<sup>TM</sup> Supercompetent cells (Stratagene #230350) and correct constructs were identified by digestion of purified plasmid DNA with PvuII. A correct construct was designated pPCRProm. The sequence of this construct is described in Figure 18.

15

#### Cloning into an LTB expression vector

Plasmid pPCRProm was digested with KpnI and BglII and the 200 bp promoter fragment was isolated from a 1% agarose gel using a QIAquick<sup>TM</sup> gel extraction kit (Qiagen) according to the manufacturer's instructions. The *nirB* promoter of pNLTB was excised by digested with KpnI and BglII (Figure 15) and the remaining 3.7kb vector fragment was isolated from a 1% agarose gel using a QIAquick<sup>TM</sup> gel extraction kit (Qiagen) according to the manufacturer's instructions. The vector was ligated to the LT promoter fragment (Sambrook *et al.*, 1989, ref 13) and the ligation mixture was used to transform *E. coli* XL10-Gold<sup>TM</sup> supercompetent cells (Stratagene #230350). Correct clones were identified by restriction enzyme analysis of purified plasmid DNA and one (designated pLLTB) was used to transform electrocompetent ETEC-PTL003.

25

## **Section 6 – Expression of LTB under the control of the LT promoter**

### **Sample preparation**

Cultures of PTL003 and PTL003-pLLTB were grown overnight in LB medium with  
5 appropriate antibiotics. Absorbance at 600nm was used to determine the  
concentration of cells in the cultures. Aliquots containing  $5 \times 10^7$  cells were  
concentrated by centrifugation and the pellets were resuspended in 10  $\mu$ l Tris-  
Glycine SDS-PAGE sample buffer (Invitrogen LC267) containing 0.1 M  
dithiothreitol.

10

### **Sample analysis**

Samples were analysed by SDS-PAGE (16) and Western blotting exactly as  
described in Section 3.

15 Results are presented in Figure 19. LTB was expressed in PTL003 under the control  
of the LTAB promoter.

Table 1: Oligonucleotides used

Name	Nucleotide sequence	Target locus; use
4712 5'-GTAACGTCTAGCGTTGATCC		<i>colA</i> ; detection of CS2 locus
4714 5'-TTCAACCTTAAAGCTTTAAAGCCT		<i>oriR6K</i> ; construction of pJCB12
4715 5'-CTACACGAACTCTGAAGATCAGCAGTTCAACC		<i>oriR6K</i> ; construction of pJCB12
4716 5'-GATCTTCAGAGTTTCGTGTAGACTTTCCTTGG		<i>mobRP4</i> ; construction of pJCB12
4717 5'-GCCACTGCAGCCTCGCAGAGCAGGATTC		<i>mobRP4</i> ; construction of pJCB12
4718 5'-GGCACTGCAGGCGTAGCACCAGGCGTTT		<i>cat</i> ; construction of pJCB12
4719 5'-TCATCCGGAGTTCCGTATGGCAAT		<i>cat</i> ; construction of pJCB12
4720 5'-TGCCATACGGAACTCCGGATGAG		<i>cat</i> ; construction of pJCB12
4721 5'-GCTTTTAAAGCTTTTAAAGTTGAA TTCGATCGGCACGTAAAGAGTTTC		<i>sacB</i> ; construction of pJCB12
4722 5'-GGCCTGCAGGCAAGACCTAAATGTG		<i>sacB</i> ; construction of pJCB12
4723 5'-GCGCTGCAGCTTTATGTGTAAAGAA		<i>efaC</i> ; detection of CFA/I
4727 5'-GCCGCATGCAITTAATTCATATATAGGG		<i>efaC</i> ; detection of CFA/I
4728 5'-GCCGTCGACTGCCATAAGGTAAACGAGC		<i>aroC</i> ; confirmation of linkage with pJCB12 and mutation
4731 5'-GAATTTTACGTGCGATGAACGG		<i>ompC</i>
4732 5'-GTACAAATAACCTACAAAAGCCCC		<i>ompF</i>
4733 5'-ACCCACACACGCTTAACGCTGG		detection of CS5
4738 5'-GGAAAGAGAGTATATCTATGTAAACGC		detection of CS5
4739 5'-CGGTCGAGTAATAAGCTGTACTCTGC		<i>css4</i> ; detection of CS6
4740 5'-TAATTCCTGCTTCATTCGGCAGCC		<i>css4</i> ; detection of CS6
4741 5'-TAGTAACCAACCAACCTGATCG		<i>aroC</i> ; confirmation of mutation
4742 5'-CTTCACACTCCAGACTATCGGC		<i>ompC</i>
4743 5'-TTCTGGCTCGGAAATTTGAACC		<i>eltB</i> ; construction of $\Delta$ LT-A
4746 5'-CGGCATGCCGCAATTTGAATGGGGG		EAST1; confirmation of linkage with pJCB12
4748 5'-AGAACTGCTGGGTATGTGGCTGG		EAST1; construction of EAST1 deletion mutation
4749 5'-GGCGTCGACGAAATGAAGGGCGGAAGTTC		EAST1; construction of EAST1 deletion mutation
4750 5'-ATGACACGAAATGTTGATGGCATCCGGGAAGC		EAST1; construction of EAST1 deletion mutation
4751 5'-GCCATCAACATTCGTGTCATGGAAGGACTAC		EAST1; construction of EAST1 deletion mutation
4752 5'-GGCGCATGCAAGATTCGGCCAGTTAGCC		EAST1; to check for linkage with pJCB12
4753 5'-GTTGGATAAGCGAAGAACGTGG		

4760 5'-GCCGTCGACTCGCGGAGCGGAAATGGC  
 4761 5'-GCCGAAATCAACTTATATCGTATGGGGC  
 4762 5'-GGAAGTTCGCTCCATTTACGGG  
 4764 5'-AATAATTAATAGTCTCTCGTAGCGG  
 4765 5'-ATTAATAGCACCCGGTACAAGCAGG  
 4766 5'-CAACAGTACTGCGATGAGTGG  
 4768 5'-CAATTGATATTTTGCAAGCTGATGG  
 4769 5'-TAGAAACGACCCCACTATAATTTC  
 4772 5'-CCGTCGACTAAAAATCACCACCACTTC  
 4773 5'-ATTCATCCTCCTTATATATCATACAAGACAATCC  
 4774 5'-GATATATAAGGAGGATGAATTATGAATAAAGTAAATTT  
 4775 5'-GTTCAATCCAGCATCAAAATGAAG  
 4777 5'-GCCGCAATGCCATTCGCCAGTCTTCAA  
 4778 5'-CCAGGCGGTACCGAACTCG  
 4779 5'-TTGAACAGAAAGAAAACTCGCAC  
 4780 5'-ATGAATCTCTCCCAACGCTCTTCC  
 4781 5'-AGTCAAATGCTCTGCATAAGTACC  
 4783 5'-GGATATATCTTTTGGTGAAGATAAG  
 4784 5'-AATAAGATGCGCTAGAAATCCC  
 4785 5'-TATGGATATATATTCAGAAGAAGAG  
 4786 5'-AATAAGACGCACTGGAAATCC  
 4789 5'-GGCTCGAGATTTCCCGACCTTAATGCG  
 4790 5'-CGGCTCGAGGACGTTGTGAGTGGCGG  
 4792 5'-GTGCTATTAATAATAATAAAGGG  
 4794 5'-TTTTCGGTCGCCGAAAGATAATA  
 4797 5'-GCGTGTCTTCAACTGTGG  
 4798 5'-CCACAGTTGAAGAACAGCGC  
 4799 5'-ATGTCGCCACGCATGACGGC  
 47100 5'-CCGGCATGCGATGCCCTGCAGATGG  
 47101 5'-GCCGTCGACTATGCTCTTCGTAGCGGAG  
 47106 5'-GAACCTTTGCTGAGTTGAAGGAGC  
 47112 5'-GGTCAGCCGGAATACCGGT  
 47113 5'-TCAGGCACAGCTAGCCGTCT  
 47114 5'-ACAGCGCCTCGAGACTATTCATGCTTTCAGGACC

*ori* pACYC184; construction of pJCB12-pACYC*Cori*  
*ori* pACYC184; construction of pJCB12-pACYC*Cori*  
 LT, construction of  $\Delta$ LT-A  
 ST strain A; construction of ST deletion mutation  
 ST strain A; construction of ST deletion mutation  
*cat*; nucleotide sequence determinations into *sacB*  
*csf4*; detection of CS4  
*csf4*; detection of CS4  
 LT, construction of  $\Delta$ LT-A  
 LT, construction of  $\Delta$ LT-A  
 LT, construction of  $\Delta$ LT-A  
 detection of EAST1  
 detection of EAST1  
  
*colC*; detection of CS2  
 detection of CS5  
*cssB*; detection of CS6  
*csvR*; detection of CS5 regulator gene  
*csvR*; detection of CS5 regulator gene  
*cfbD*; detection of CFA/I regulatory gene.  
*cfbD*; detection of CFA/I regulatory gene  
*parDE*; construction of pJCB12-ASTI<sup>B</sup>::*parDE*  
*parDE*; construction of pJCB12-ASTI<sup>B</sup>::*parDE*  
 STI<sup>B</sup>, nucleotide sequencing downstream of STI in Strain B  
 STI, sequencing upstream of STI and confirmation of linkage  
 STI<sup>B</sup>, nucleotide sequencing downstream of STI in Strain B  
 STI<sup>B</sup>, nucleotide sequencing downstream of STI in Strain B  
 STI<sup>B</sup>, construction of pJCB12-ASTI<sup>B</sup>  
 STI<sup>B</sup>, construction of pJCB12-ASTI<sup>B</sup>  
 STI<sup>B</sup>, construction of pJCB12-ASTI<sup>B</sup>  
 STI<sup>E</sup>, nucleotide sequencing downstream of STI in Strain E  
 STI<sup>E</sup>, construction of pJCB12-ASTI<sup>E</sup>  
 STI<sup>E</sup>, confirmation of linkage of STI in Strain E  
 STI<sup>B</sup>, construction of pJCB12-ASTI<sup>B</sup>

47115	5'-GAATAGTCTCGAGGCGCTGTTCTTCAACTGTGG	STI <sup>B</sup> , construction of pJCB12-ΔSTI <sup>B</sup>
47116	5'-GGGTCTAGACACAAACAATAACGGAGCCGTG	<i>aroC</i> ; construction of pJCB12-Δ <i>aroC</i> <sup>d</sup>
47117	5'-GGCGAGCTCGGAATATCAGTCTTCACATCGG	<i>aroC</i> ; construction of pJCB12-Δ <i>aroC</i> <sup>d</sup>
47118	5'-CCACGCCCTTTCACCCACCCGCCGATAATCGC	<i>aroC</i> ; construction of pJCB12-Δ <i>aroC</i> <sup>d</sup>
47119	5'-CGCGGCGGTGGGTGAAAGCGGTGGAAATTGGC	<i>aroC</i> ; construction of pJCB12-Δ <i>aroC</i> <sup>d</sup>
47120	5'-CATCAGAAATCACTATTCATGCTTCAGGACCCAC	STI <sup>E</sup> , construction of pJCB12-ΔSTI <sup>E</sup>
47121	5'-CATGAATAGTGAATTCGTGATGATGCTGTAAACG	STI <sup>E</sup> , construction of pJCB12-ΔSTI <sup>E</sup>
4917	5'-ATCAACGGTGGTATATCCAGT	<i>cat</i> of pJCB12; confirmation of linkage.
Bfor	5'-ACGTAGATCTTTATGAATAAAGTAAATTTTATG	LT-B;
BglIIIFOR	5'-CCCAGATCTATATGCATAAAATTAATCTATTACTAAG	<i>cfaB</i> ; detection of CFA/I
BglIIImodREV	5'-CACTTGGTAAAGACCTAATTAGAGCCGC	<i>cfaB</i> ; detection of CFA/I
Brev	5'-GTACGCTAGCCATGTATCTCATTAGCTG	LT-B;
CS3-02	5'-TTGTCGAAGTAAATTGTTATA	detection of CS3 genes
CS3-03	5'-GTGAATGTATGAGGGATTCTGA	detection of CS3 genes
CS3-06	5'-CTAAATGTTCTGTTACCTTCAGTGG	detection of CS3 genes
EST-01	5'-CATGTTCCGGAGGTAATATGAA	detection of ST gene
LT-04	5'-CATCGCCATTATATGCAAATGGCG	<i>eltA</i> ; detection of LT genes
LT-05	5'-ACTGATTGCCGCAATTGAATTGGG	<i>eltB</i> ; detection of LT genes
Pfor	5'-CCGGTACCATGATTCATGTATACACC	LT promoter
Prev	5'-ACGTAGATCTACTTATATATCATACAAG	LT promoter
R6K-01	5'-GTGACACAGGAACACTTAACGGC	<i>oriRK</i> ; confirmation of linkage
RNS-03	5'-ACATCATAGCGATGGCATCAA	<i>rns</i> ; detection of CFA/II regulatory gene
RNS-04	5'-TATTTCAATTCAAGTTCGCATCGC	<i>rns</i> ; detection of CFA/II regulatory gene
ST-01	5'-CATGACGGGAGGTAAACATGA	Detection of ST <sub>1681</sub>
ST-02	5'-TATGCTTTTAAATAACATCC	Detection of ST <sub>1681</sub>
TT1	5'-ATCTGTTTGTGAGCTCAGCAATCTATTGCAACC	<i>ompF</i>
TT20	5'-ATGCGCGGAGAGCTCAACGAGGTGCGCACTTTG	<i>aroC</i>
TT33	5'-TTGTAGCACTTTCACGGTAG	<i>ompF</i> ; nucleotide sequence determinations across Δ <i>ompF</i>
TT35	5'-GATGGTGTGTTTATGCTC	<i>aroC</i> ; nucleotide sequence determinations across Δ <i>aroC</i>
TT38	5'-GGAGAAATGGACTTGCCGAC	<i>ompC</i> ; nucleotide sequence determinations across Δ <i>ompC</i>
47104	5'-TTATTGATGGAAGCTCAGGAGG	
47105	5'-TAACGCCCTGCTCAACATTCCC	
4792	5'-GTGCTATTATAATATAAAGGG	
4730	5'-TTCTTCACGAACTAATTGAGTG	

**Table 2****GenBank Accession numbers for sequence data**

5	EAST1 ( <i>astA</i> )	AF143819
	ST ( <i>estA</i> )	M18346
	LT-A ( <i>eltA</i> )	V00275
	LT-B ( <i>eltB</i> )	M17874
10	CFA/I operon	M55661
	CS2 operon	Z47800
	CS3 operon	X16944
	CS4 operon	AF296132
	CS5 operon	AJ224079
15	CS6 operon	U04844
	<i>cfaD</i>	M55609
	<i>csvR</i>	X60106
	<i>rns</i>	J04166
	<i>parDE</i> RK2	L05507
20	<i>sacB</i>	X02730
	<i>oriR6K</i>	M65025
	<i>mobRP4</i>	X54459
	<i>cat</i>	V00622

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### CLAIMS

1. A bacterial cell which expresses colonization factor antigen CFA/I from a native plasmid but does not express heat stable toxin (ST).  
5
2. A bacterial cell which expresses colonization factor antigen CS5 from a native plasmid and/or expresses colonization factor antigen CS6 from a native plasmid, but does not express heat stable toxin (ST).
- 10 3. A cell according to claim 1 or 2 which is an *Escherichia coli* cell.
4. A cell according to claim 1, 2 or 3 wherein the plasmid is an enterotoxigenic *E.coli* plasmid in which the ST gene is inactivated or deleted.
- 15 5. A cell according to any one of the preceding claims wherein the plasmid contains a deletion of all or part of the ST gene.
6. A cell according to any one of the preceding claims which is obtainable by a method comprising deletion of all or part of the ST gene with a suicide vector.  
20
7. A cell according to any one of the preceding claims which does not express heat labile toxin (LT).
8. A cell according to any one of the preceding claims which does not express  
25 EAST1.
9. A cell according to any one of the preceding claims which does not express an antibiotic resistance gene.
- 30 10. A cell according to claim 7, 8 or 9 which is obtainable by a method comprising site-directed deletion or inactivation of the LT gene, the EAST1 gene and/or one or more antibiotic resistance genes.

11. A cell according to any one of the preceding claims wherein the plasmid contains an element which enhances its stability.
12. A cell according to claim 11 wherein said element is a toxin-antitoxin  
5 element or a recombinase recognition element.
13. A cell according to claim 12 wherein the stability element is *parDE* or *crs*.
14. An *Escherichia coli* cell deposited with the European Collection of Cell  
10 Cultures (ECACC) under accession number 01090303, number 01090304, number 01090305, number 01090306, number 02082964, number 02082965, number 02082966, number 02082967 or number 02082968; or a descendent of a said cell.
15. A cell according to any one of the preceding claims which is further  
15 attenuated by a site-directed deletion or inactivation of a gene.
16. A cell according to claim 15 which is further attenuated by deletion or inactivation of one or more of *aroA*, *aroC*, *aroD*, *aroE*, *pur*, *htrA*, *ompC*, *ompF*, *ompR*, *cya*, *crp*, *phoP*, *surA*, *rfaY*, *dksA*, *hupA*, *sipC* and *clpB*.  
20
17. A cell according to claim 15 which is further attenuated by deletion or inactivation of at least one *aro* gene and at least one *omp* gene.
18. A cell according to claim 15 which is further attenuated by deletion or  
25 inactivation of at least one *aro* gene and the *htrA* gene.
19. A cell according to claim 15 which is further attenuated by deletion or inactivation of each of *aroC*, *ompF* and *ompC*.
- 30 20. A cell according to any one of the preceding claims which expresses a heterologous antigen.

21. A cell according to claim 20 wherein the heterologous antigen is an *E.coli* antigen.
22. A cell according to claim 21 wherein the heterologous antigen is an *E.coli* colonization factor antigen (CFA).
23. A cell according to claim 21 wherein the heterologous antigen is a non-toxic component or form of LT.
24. A cell according to claim 23 wherein the non-toxic component of LT is the B subunit.
25. A native enterotoxigenic *E.coli* plasmid in which the gene encoding ST toxin is deleted or inactivated and which encodes:
- colonization factor antigen CFA/I; or
  - colonization factor antigen CS5 and/or colonization factor antigen CS6.
26. A vaccine against diarrhoea comprising a cell as claimed in any one of claims 1 to 24 and a pharmaceutically acceptable carrier or diluent.
27. A vaccine against diarrhoea comprising both a cell as claimed in claim 1 and a cell as claimed in claim 2, and a pharmaceutically acceptable carrier or diluent.
28. A vaccine according to claim 26 or 27 which further comprises a cell which expresses colonization factor antigen CFA/II.
29. A vaccine according to claim 26, 27 or 28 which comprises:
- (i) a cell which expresses CFA/I as claimed in any one of claims 1 and 3 to 24;
  - (ii) a cell which expresses CS5 and CS6 as claimed in any one of claims 2 to 24;
  - (iii) a cell which expresses CS4 and CS6;
  - (iv) a cell which expresses CS2 and CS3; and
  - (v) a cell which expresses CS1 and CS3.

30. A vaccine as claimed in claim 29 wherein:
- (i) the cell which expresses CFA/I is that deposited with ECACC under accession number 01090303 or 02082967, or a descendent thereof;
  - (ii) the cell which expresses CS5 and CS6 is that deposited with ECACC under  
5 accession number 01090305 or 02082968, or a descendent thereof;
  - (iii) the cell which expresses CS4 and CS6 is that deposited with ECACC under accession number 01090306 or 02082966, or a descendent thereof;
  - (iv) the cell which expresses CS2 and CS3 is that deposited with ECACC under accession number 01090304 or 02082964, or a descendent thereof; and
  - 10 (v) the cell which expresses CS1 and CS3 is that deposited with ECACC under accession number 01090302 or 02082965, or a descendent thereof
31. A cell as claimed in any one of claims 1 to 24 or a vaccine as claimed in any one of claims 26 to 30 for use in a method of vaccination against diarrhoea.
- 15 32. Use of a cell as claimed in any one of claims 1 to 24 or a combination of cells as set forth in any one of claims 27 to 30 for the manufacture of a medicament for vaccination against diarrhoea.
- 20 33. A method of vaccinating a mammal against diarrhoea, which comprises administering to the mammal a cell as claimed in any one of claims 1 to 24 or a vaccine as claimed in any one of claims 26 to 30.
- 25 34. A suicide vector which is less than 5 kb in size and comprises the *sacB* region which codes for a product that is toxic to bacteria when grown on sucrose, in which region the IS1 insertion sequence is deleted or inactivated.
- 30 35. A vector according to claim 34 which further comprises a transfer origin that directs conjugative transfer of the vector from one bacterial strain to another.
36. A vector according to claim 35 wherein the transfer origin is *mobRP4*.



37. A vector according to claim 34, 35 or 36 which further comprises an origin of replication.
38. A vector according to claim 37 wherein the origin of replication is *oriR6K*  
5 which requires the *pir* gene for replication.
39. A vector according to any one of claims 34 to 38 which further comprises a selectable maker.
- 10 40. A vector according to claim 39 wherein the selectable marker is the *cat* gene which codes for chloramphenicol acetyltransferase and confers resistance to chloramphenicol.
41. A vector according to any one of claims 34 to 40 which further comprises a  
15 cloning site comprising at least one restriction enzyme site unique in the vector.
42. A vector according to any one of claims 34 to 41 which is about 3kb in size.
43. A vector according to claim 41 which comprises sequence of a target gene in  
20 the cloning site.
44. A vector according to claim 43 which comprises a wild-type or inactivated *E. coli* toxin gene in the cloning site.
- 25 45. A vector according to claim 44 which contains a wild-type or inactivated ST gene in the cloning site.
46. A method for producing a bacterial cell in which a target gene is deleted, inactivated or replaced, which method comprises transferring a vector as claimed in  
30 claim 43, 44 or 45 into a bacterial cell containing the target gene and selecting for a cell in which the target gene has been deleted, inactivated or replaced.

47. A method according to claim 46, which comprises carrying out PCR to select for a cell in which the vector has correctly targeted to the target gene, wherein one of the primers used in the PCR hybridizes to vector sequence adjacent to the cloning site and the other hybridizes to a site in the cellular DNA adjacent to the target gene,  
5 and wherein a positive PCR indicates that the vector has targeted to the target gene.

48. A method according to claim 46 or 47 which comprises selecting for a cell from which the vector has been excised by growing the cell in medium supplemented with sucrose from which NaCl is absent.

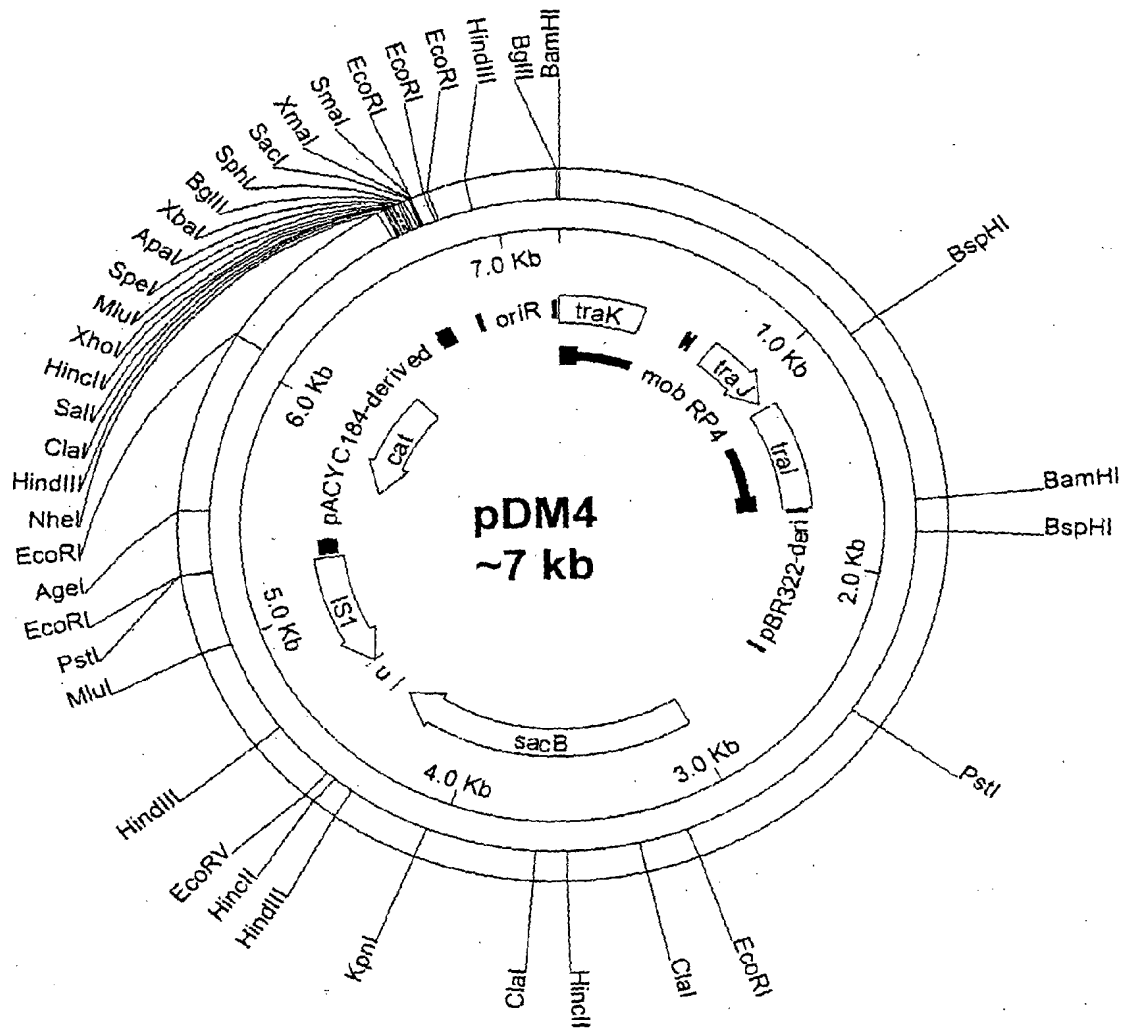


Figure 1.

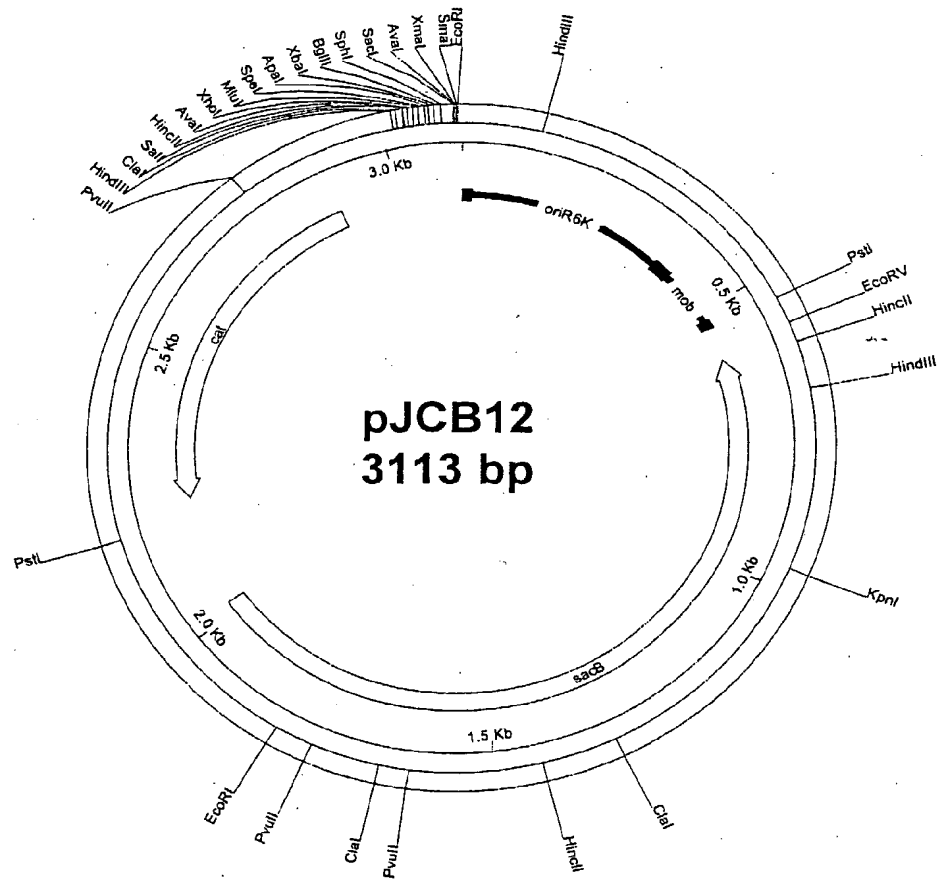
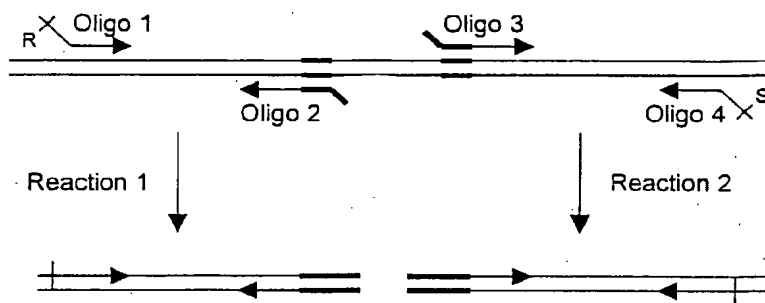


Figure 2.

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## Step 1



## Step 2

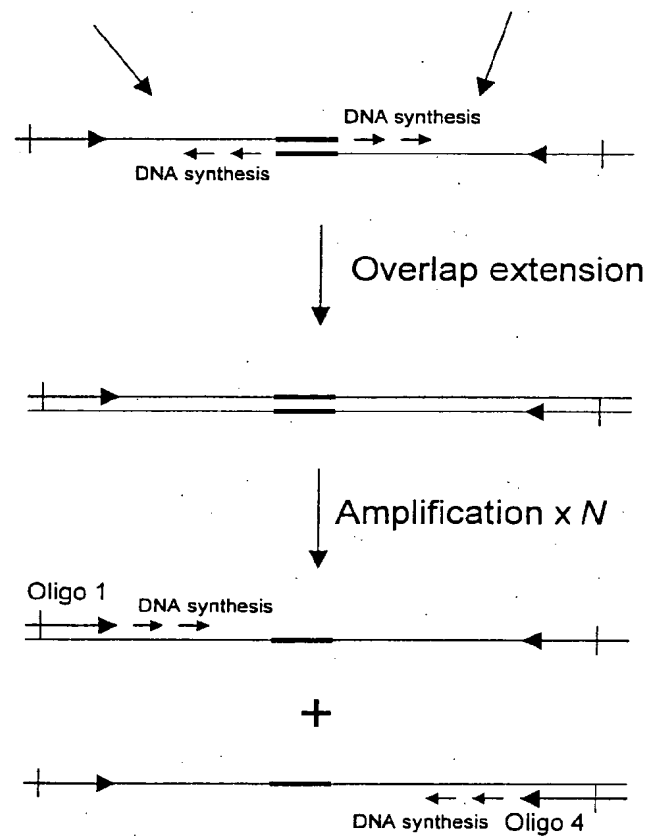
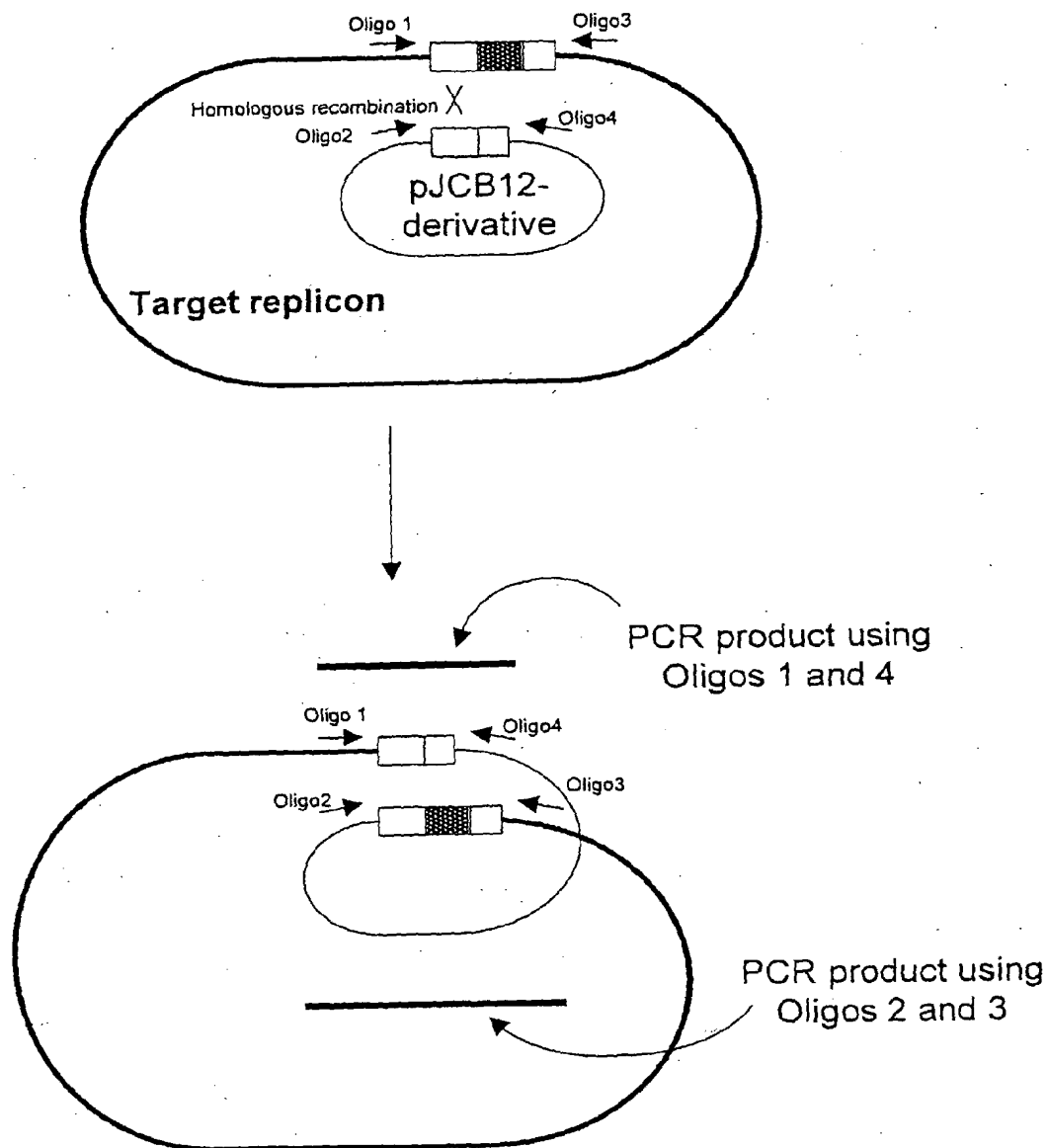


Figure 3.

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**Figure 4.**

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Figure 5.

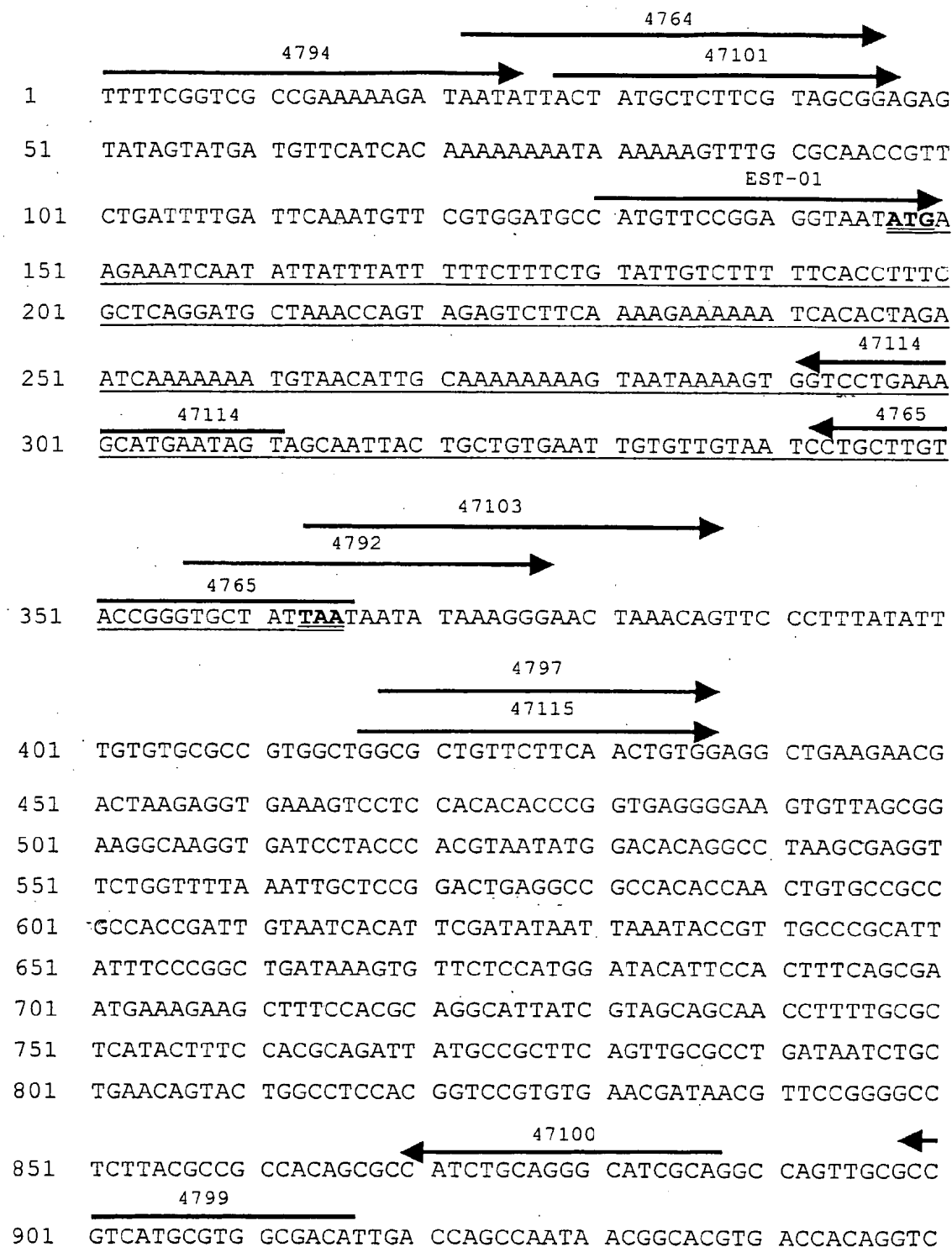


Figure 6:

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1 GTTGGGTTGA GCCTGTACAT AGATTGTGT AATTGCCTGA TTTTGATATG ➤  
 ——— 4775 ——— ➤  
 51 TTCAATCCAG CATCAAATGA AGGTTAATTT ATGGACGAAA AACAGTTACA  
 101 GACTCTGGCT AACGAACTGG CCAAAAACCT CAAAACCCCT GAAGACCTCA  
 151 GTCAGTTTGA TCGGCTGCTG AAAAAGCTCA GCGTTGAAGC CGCTCTCAAT  
 201 GCAGAGATGA CACACCATCT TGGGTATGAG AAAAATCAGT CCAGACCAGG  
 251 AGCTAACTCC CGCAACGGTT ATTCCACAAA GACCGTTATC ACAGGCGACG  
 301 GTCCACTGGA ACTGCGTACT CCGCGCGATC GTGACGGTAC CTTCGAACCA  
 351 CAACTGGTAA AGAAAAATCA GACCCGTATT ACCGGGATGG ATAACCAGAT  
 401 CCTCTCGTTG TATGCCAAAG GGATGACCAC CCGTGAGATA GCCGCTGCGT  
 451 TCAAAGAACT GTATGACGCA GATGTTTCAC CGGCACTGAT ATCAAAGGTT  
 501 ACCGATGCCG TGATGGAGCA GGTTGTAGAA TGGCAAAACC GACCACTGGA  
 551 TGCTGTTTAC CCCATTGTTT ATCTTGA CTG TATCGTCCTG AAAGTTCGGC  
 601 AGGACAGTCG CGTCATCAAC AAATCGGTGT TCCTGGCACT GGGCATCAAT  
 ——— 4748 ——— ➤ — 4749 ➤  
 651 ATCGAAGGTC AGAAAGAACT GCTGGGTA TGCTGGCCG AAAATGAAGG  
 ——— ➤  
 701 GGCGAAGTTC TGGCTCAATG TGCTGACTGA ACTGAAAAAC CGCGGTCTGA  
 ——— 4750 ———  
 751 ACGATATCCT CATCGCCTGT GTGGATGGCC TGAAAGGCTT CCCGGATGCC  
 ———  
 801 ATCAACACAG TATATCCGAA GGCCCGCATC CAGTTATGCA TCGTGCATAT  
 ——— 4751 ——— ➤  
 851 GGTGCGCAAC AGCCTGCGCT TCGTGTCATG GAAGGACTAC AAAGCCGTCA  
 901 CTCGCGACCT GAAAGCGATT TATCAGGCTC CCACGGAAGA GGCAGGTCAG  
 ———  
 951 CAGGCCCTGG AAGCGTTCGC TGCGGCCTGG GACAGTCGCT ATCCTCAGAT  
 ——— 4752 ——— — 4753 —  
 1001 AAGCCGAAGC TGGCAGGCTA ACTGGCCGAA TCTTGCCACG TTCTTCGCTT  
 ———  
 1051 ATCCAACGGA CATCCGCAAA GTGATCTATA CGACGAATGC CATCGAGTCG  
 1101 CTAAACAGCG TGATCCGCCA TGCGATCAAA AAGCGTAAAG TGTTCCCGAC  
 1151 AGACGACTCG GTGAAAAAAG TGGTGTGGCT GGCAATCCAG TCTGCGTCCC  
 ——— 4777 ———  
 1201 GGAAATGGAC GATGCCGTTG AAGGACTGGC GAATGGCAAT GAGCCGCTTT  
 1251 ATTATCGAGT TCGGTGACCG CCTGGACGGT CACTTCTGAG AAAGGCATTT  
 1301 ACACAGAATA CTAAACAGGC TCGTTGGGTT



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Figure 7:

```
1   GGTGACCCG CTGAAATGCA TTCTTTGCNG AAGTCAGATG TGGTNNACGG
51  GACTGAAGAG GGGCTACCGT CTGGCAGAGC TGGTCGGGAT GCATGAGCCA
    — 4762 —>
101 CTGGCGCGAC AGCGGGTGTG CGGCTGANAG CCGCAGAGGG GAAGTTGCGT
    — 4762 —>
151 CCATTTTACG GGGGATGGAG CCAAAAAAAC CGCCAATCAT ACCCTGTATC
201 AATCAGAGTC ATCCTGTTTA ATAGTCATTT CTGTTTATAT GGTGCACAAG
251 GAGTGTGAA GCAACATCCG TTTTGTGGTG TTTTTTAAAT CTTTTTGGGA
    — 4772 —>
301 TTTTAATTCC TATCGATGAA CAGGCGTTTC AGCGGTCGGG ACTAAAAATC
    — 4772 —>
351 ACCACCACTT CGGGTCATCC GCCTTCATCT CCGCTTCTGT TTCGTATAAA
401 TCAAAACGAC GGCAGGTATG GCAGAACGTG ACGTATTCGT GCGGTGAATC
451 CGGGTACATT TTGTGGAAGG TTTCCAGAA ACAGACCGTG CAGGAGGGAT
501 GACCGGCGAT AAAGTCTGTA AATACGGACT GATAAGGGTG ATTATTGGCT
551 CTGGCGACGG CTTTCAGAAC CTCTTTCACC ATTCTGGTGT GGACTTCTG
601 GTGCTCCAGG TTGTGTGACA TGGGAACTCA TTCTGGATGG TTAATCTGAA
651 AGCCCATATT CTGCCCCCCC CCGATTGCA GCCGCCAGGC TGCCGTGGTT
    — Pfor —>
701 CAAGTCGCGA CTAATAAAAA TAATCAGGTT GCCATGATTC AATGTACACC
751 TTTCTCACAT TCGTCTCCGG CATGAAAACG ATGCACTCTT CCTTTATCGC
801 TTTCACTACA CATTTTATCC TCGCATGGAT GTTTATAAAA AACATGATTG
851 ACATCATGTT GCATATATGT TAAATAAAAC AAGTGGCGTT ATCTTTTCC
    — 4773 —<
    — Prev —<
901 GGATTGTCTT CTTGTATGAT ATATAAGTTT TCCTCGATGA AAAA
```

Figure 8:

AATAAAAAAG	TTTGCGCAAC	CGTTCGTGATT	TTGATACAAA	TGTTTCGTGGA	50
EST-01					
TGCCATGTTC	CGGAGGTAAT	<u>ATGAAGAAAT</u>	CAATATTATT	TATTTTTCTT	100
Start codon					
TCTGTATTGT	CTTTTTCACC	TTTCGCTCAG	GATGCTAAAC	CAGTAGAGTC	150
TTCAAAAGAA	AAAATCACAC	TAGAATCAAA	AAAATGTAAC	ATTGCAAAAA	200
47120					
AAAGTAATAA	AAGTGGTCCT	GAAAGCATGA	ATAGTAGCAA	TTACTGCTGT	250
4765					
GAATTGTGTT	GTAATCCTGC	TTGTACCGGG	TGCTATTAAT	AATATAAAGG	300
Stop codon 47121					
GAACTAAACA	GTTCCCTTTA	TATTTGGTCT	GATTCTGATG	ATGTCTGTAA	350
47106					
CGTATGTACC	TGTTGCTTTG	TTGAATAAAT	CGAACTTTTG	CTGAGTTGAA	400
GGAGCAGAGC	ACGCATCATC	CGGCAACATG	AGTCGTTCCA	TGGCAAAGCA	450
GAAGTTCAGA	ATCACCAACT	GGCGCAGCCA	CAACAAAGCC	CTTATCCTCC	500
GTGGCACCAT	CACTTTCTGG	CGGGATGGCG	AGGCAATTCA	GGCCTGGTAT	550
GAGTCAGCAA	CCCCCTCATC	ACGGGGACGA	CCTCAGCGTT	ATTCTGACCT	600
47112					
TGCTATTACC	ACTTTTTTTG	TGATTAAACG	CGTATTCCGG	CTGACCGACC	650
CTGCGGGCTT	GAAGTGCCCC	CGAAAGTTGG	ACAGTTTATT	GTTAGACGGC	700
47113					
TAGCTGTGCC	TGAGTCCGGT	ATTCTTACCC	GGGCTCAGGC	CATTTAACCT	750
TGAGCTAATC	CGCTCATTGT	TAATCGATAT	ACTCCTCGAC	TACTCTTCGA	800
CGCTCTTTTG	TACTATAATA	AGCTTTTTTCT	GCTTCAGCCG	GGACTCCATA	850
CCGCGCTGAT	GCCTGCCGTT	CCATTCCCTGC	TGTGTAAGCG	TCAACGGAGC	900
ACCGTATTGA	CGCTCATTTA	TTGGTGAGTA	CTACGTTCCA	TGGCAGGAGT	950
TCGCCAACTC	GGTTGGAAGG	CCATTCCGGC	AGTACGCTCA	GAATATGGCG	1000
CAGATACGCT	TCCGGATCGA	TACCGTTCAG	ACGGCAGGTG	CCGATCATGC	1050
CCGTACAGCA	GTGCTCCACG	CTCGCCCGCC	GTGATCGCTA	CCGAAGAACA	1100
CGTAATTTTT	CTTTCCCGAG	ACCAGACTTG	CACGAAAGCG	CTCTTTCCGC	1150
TGGTATTGTC	CGCTCTGCCA	GACCGTATTA	CAC		1183

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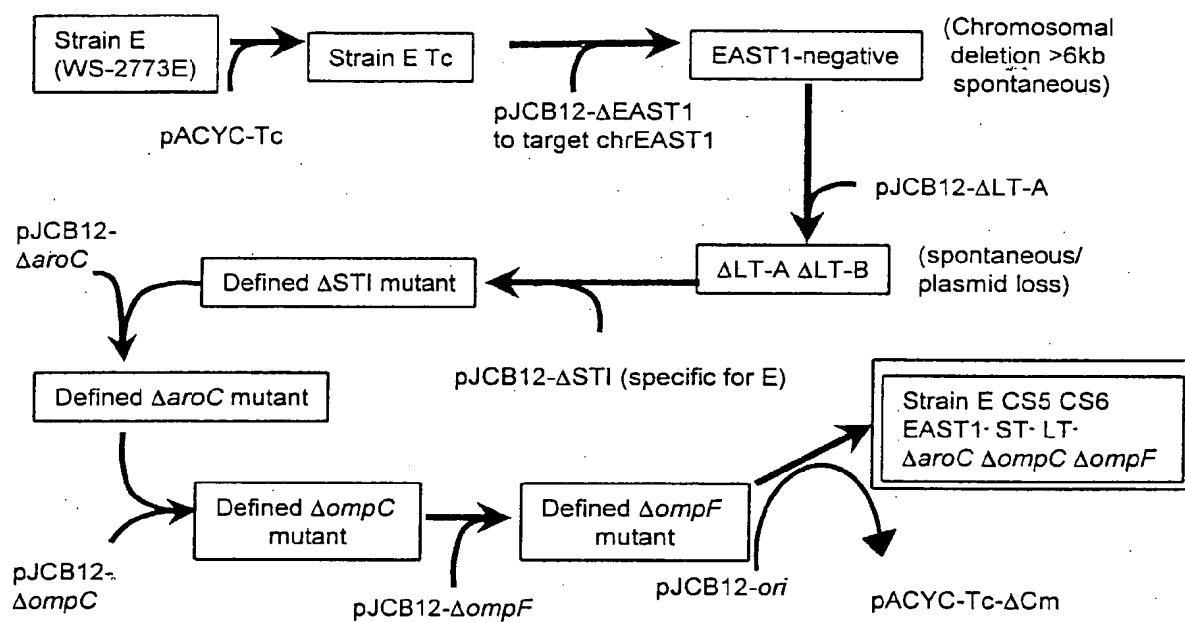


Figure 9.

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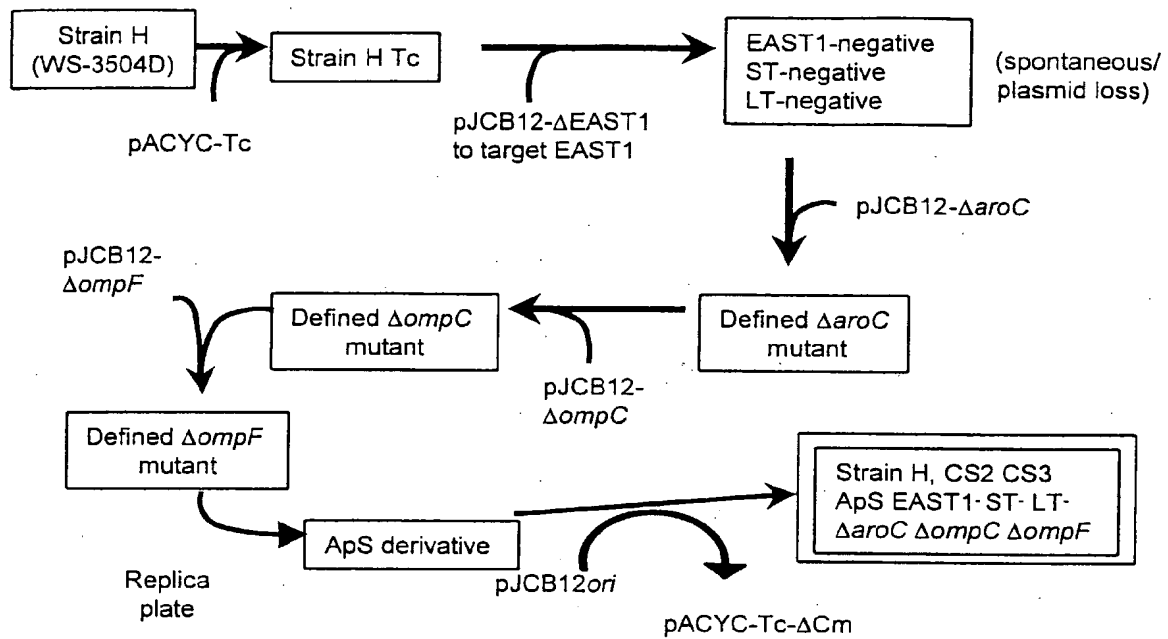


Figure 10.

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Figure 11

1 CTGGAGGAAT ACGTGGATAA AATTTTCGTT GATGAAGCAG TAAATGAGCT  
51 GCAAACCATT CAGGACATGT TGCCTGGTC GGTGAGCCGC TTCAGCGCGG  
101 CAAATATCTG GTACGGTCAC GGTACCGATA ACCCGTGGGA TGAAGCCGTA  
151 CAGCTGGTGT TGCCTTCGCT CTACCTGCCG CTGGATATTC CGGAAGATAT  
201 GCGCACCGCG CGTCTGACCT CCAGCGAAAA ACACCGTATT GTTGAACGCG  
251 TGATCCGCCG CGTCAATGAA CGCATTCCGG TGGCTTACCT GACCAACAAA  
301 GCGTGGTTCT GCGGCCATGA ATTTTACGTC GATGAACGCG TGCTGGTGCC  
351 GCGCTCGCCG ATTGGTGAAC TGATCAACAA TAAATTTGCC GGACTTATCA  
401 GCAAGCAACC GCAGCATATT TTAGATATGT GTACTGGTAG CGGCTGCATC  
451 GCCATTGCCT GTGCTTATGC CTTCCCGGAT GCAGAAGTCG ACGCGGTGGA  
501 TATCTCTCCA GACGCGCTGG CGGTTGCTGA ACAGAACATC GAAGAACACG  
551 GTCTGATCCA CAACGTCATT CCGATTCTGT CCGATCTGTT CCGCGACTTG  
601 CCGAAAGTGC AGTACGACCT GATTGTCACT AACCCGCCGT ATGTCGATGC  
651 GGAAGATATG TCCGACCTGC CAAACGAATA CCGCCACGAG CCGGAACTGG  
701 GCCTGGCATC TGGCACTGAC GGCCTGAAAC TGACGCGTCG CATTCTCGGT  
751 AACGCGGCAG ATTACCTTGC TGATGATGGC GTGTTGATTT GTGAAGTCGG  
801 CAACAGCATG GTACATCTTA TGGAAACAATA TCCGGATGTT CCGTTCACCT  
851 GGCTGGAGTT TGATAACGGC GGCGATGGTG TGTTTATGCT CACCAAAGAG  
901 CAGCTTATTG CCGCACGAGA ACATTTTCGCG ATTTATAAAG ATTAAGTAA  
951 CACGCAAACA CAACAATAAC GGAGCCGTGA TGGCTGGAAA CACAATTGGA  
1001 CAACTCTTTC GCGTAACCAC CTTCGGCGAA TCGCACGGGC TGGCGCTCGG  
1051 CTGCATCGTC GATGGTGTTT CGCCAGGCAT TCCGCTGACG GAAGCGGACC  
1101 TGCAACATGA CCTCGACCGT CGTCGCCCTG GGACATCGCG CTATACCACC  
1151 CAGCGCCGCG AGCCGGATCA GGTCAAAATT CTCTCCGGTG TTTTTGAAGG  
1201 CGTTACTACC GGCACCAGCA TTGGCTTGTT GATCGAAAAC ACTGACCAGC  
1251 GCTCTCAGGA TTACAGTGCG ATTAAGGACG TTTCCCGTCC AGGCCATGCC  
1301 GATTACACCT ACGAACAAAA ATACGGTCTG CGCGATTATC GCGGCGGTGG  
1351 ACGTTCTTCC GCCCGCGAAA CCGCCATGCG CGTGGCGGCA GGAGCTATTG  
1401 CCAAAAATA TCTCGCCGAG AAATTTGGTA TGAAATCCG TGGCTGCCTG  
1451 ACCCAGATGG GCGACATTCC GCTGGATATC AAAGACTGGT CGCAGGTCGA  
1501 GCAAAATCCG TTTTTTTGCC CGGACCCCGA CAAAATCGAC GCGTTAGACG  
1551 AGTTGATGCG TGCGCTGAAA AAAGAGGGCG ACTCCATCGG CGCTAAAGTC  
1601 ACCGTTGTTG CCAGTGGCGT TCCTGCCCGA CTTGGCGAGC CGGTCTTTGA  
1651 CCGCCTGGAT GCTGACATCG CCCATGCGCT GATGAGCATC AACGCGGTGA

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— 47119 →

1701 AAGGCGTGGA AATTGGCGAC GGCTTTGACG TGGTGGCGCT GCGCGGCAGC  
1751 CAGAACCGCG ATGAAATCAC CAAAGACGGT TTCCAGAGCA ACCATGCGGG  
1801 CGGCATTCTC GGCGGTATCA GCAGCGGGCA GCAAATCATT GCCCATATGG  
1851 CGCTGAAACC GACCTCCAGC ATTACCGTGC CGGGTCGTAC CATTAAACCGC  
1901 TTTGGCGAAG AAGTTGAGAT GATCACCAAA GGCCGTCACG ATCCCTGTGT  
1951 CGGGATCCGC GCAGTGCCGA TCGCAGAAGC GATGCTGGCG ATCGTTTAA  
2001 TGGATCACCT GTTACGGCAA CGGGCGCAA ATGCCGATGT GAAGACTGAT  
← 47117 —  
2051 ATTCCACGCT GGTAAAAAAT GAATAAAACC GCGATTGCGC TGCTGGCTCT  
2101 GCTTGCCAGT AGCGCCAGCC TGGCAGCGAC GCCGTGGCAA AAAATAACCC  
2151 AACCTGTGCC GGGTAGCGCA CAATCGATAG GCAGTTTTTC TAATGGCTGT  
2201 ATTGTCGGCG CTGACACGCT GCCGATACAG TCCGAACATT ATCAGGTCAT  
2251 GCGTACCGAT CAGCGTCGCT ATTTGCGTCA CCCGGATCTG GTGATGTTTA  
2301 TCCAGCGTCT GAGTAGCCAG GTGAGCAATC TGGGCATGGG TACGGTGCTG  
2351 ATTGGCGATA TGGGGATGCC CGCTGGTGGG CGTTTCAACG GCGGTCATGC  
2401 CAGCCACCAG ACCGGACTGG ATGTCGATAT CTTTCTGCAA CTGCCGAAAA  
2451 CTCGCTGGAC CTCCGCGCAG CTCTTGCGCC CGCAAGCACT GGACTTAGTT  
2501 TCCCGCGACG GTAAACACGT TGTCTCCACG CTGTGGAAGC CAGAAATTTT  
2551 CAGCTTGATC AAACCTCGCCG CCCAGGACAA AGACGTCACG CGCATTTTTG  
2601 TTAATCCGGC GATTAAACAA CAACTTTGCC TTGATGCGGG CACCGATCGC  
← TT20 —  
2651 GACTGGTTGC GCAAAGTGCG ACCCTGGTTC CAGCATCGCG CGCATATGCA  
← 4742 —  
2701 TGTACGATTA CGTTGCCCTG CCGATAGTCT GGAGTGTGAA GATCAACCTT  
2751 TACCGCCATC AGGCGATGGT TGCGGGGCAG AACTGCAAAG CTGGTTTGAA  
2801 CCTCCAAAAC CGGGAACAAC AAAGCCTGAG AAGAAGACAC CGCCTCCGTT  
2851 GCCGCCTTCC TGCCAGGCGC TACTGGATGA GCACGTGATC TAATGGAAAC  
2901 GTTTAATAGC CTGTTTATGG TTTCCCCGCT GTTGCTGGGA GTTCTCTTTT  
2951 TTGTCGCCAT GCTGGCGGGA TTTATCGACT CGATTGCCGG TGGCGGTGGG  
3001 TTAATCACC AATCCGGCATT GATGGCAGCG GGGATGTCTC CCGCTAATGC  
3051 GCTGGCAACC AATAAACTGC AAGCCTGCGG CGGCTCTATT TCCGCTACTA

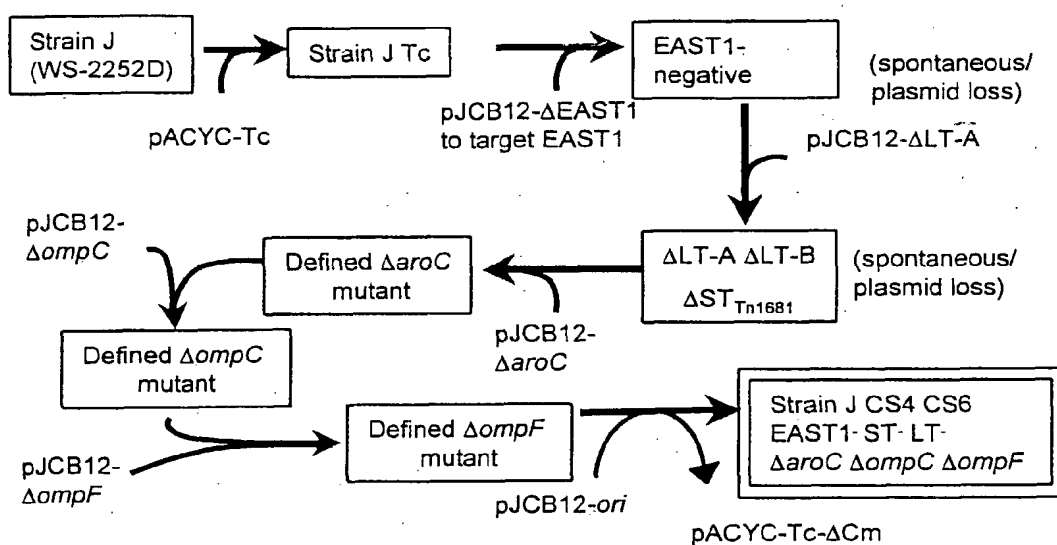


Figure 12.

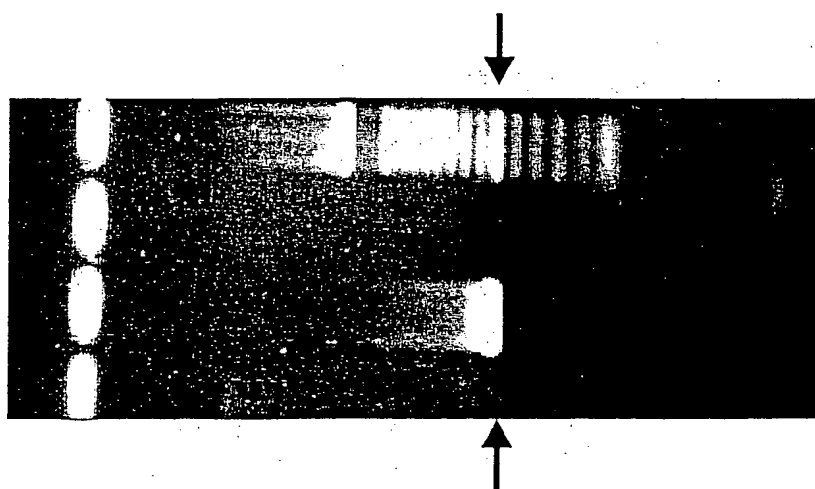


Figure 13



**Figure 14**

```
1  ATGAATAAAG TAAAATTTTA TGTTTTATTT ACGGCGTTAC TATCCTCTCT
51  ATGTGCACAC GGAGCTCCCC AGTCTATTAC AGAACTATGT TCGGAATATC
101 GCAACACACA AATATATACG ATAAATGACA AAATACTATC ATATACGGAA
151 TCGATGGCAG GCAAAAGAGA AATGGTTATC ATTACATTTA AGAGCGGCGC
201 AACATTTCAG GTCGAAGTCC CGGGCAGTCA ACATATAGAC TCCCCAAAAA
251 AAGCCATTGA AAGGATGAAG GACACATTAA GAATCGCATA TCTGACCGAG
301 ACCAAAATTG ATAAATTATG TGTATGGAAT AATAAAACCC CCAATTCAAT
351 TCGGGCAATC AGTATGGAAA ACTAG
```

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Figure 15

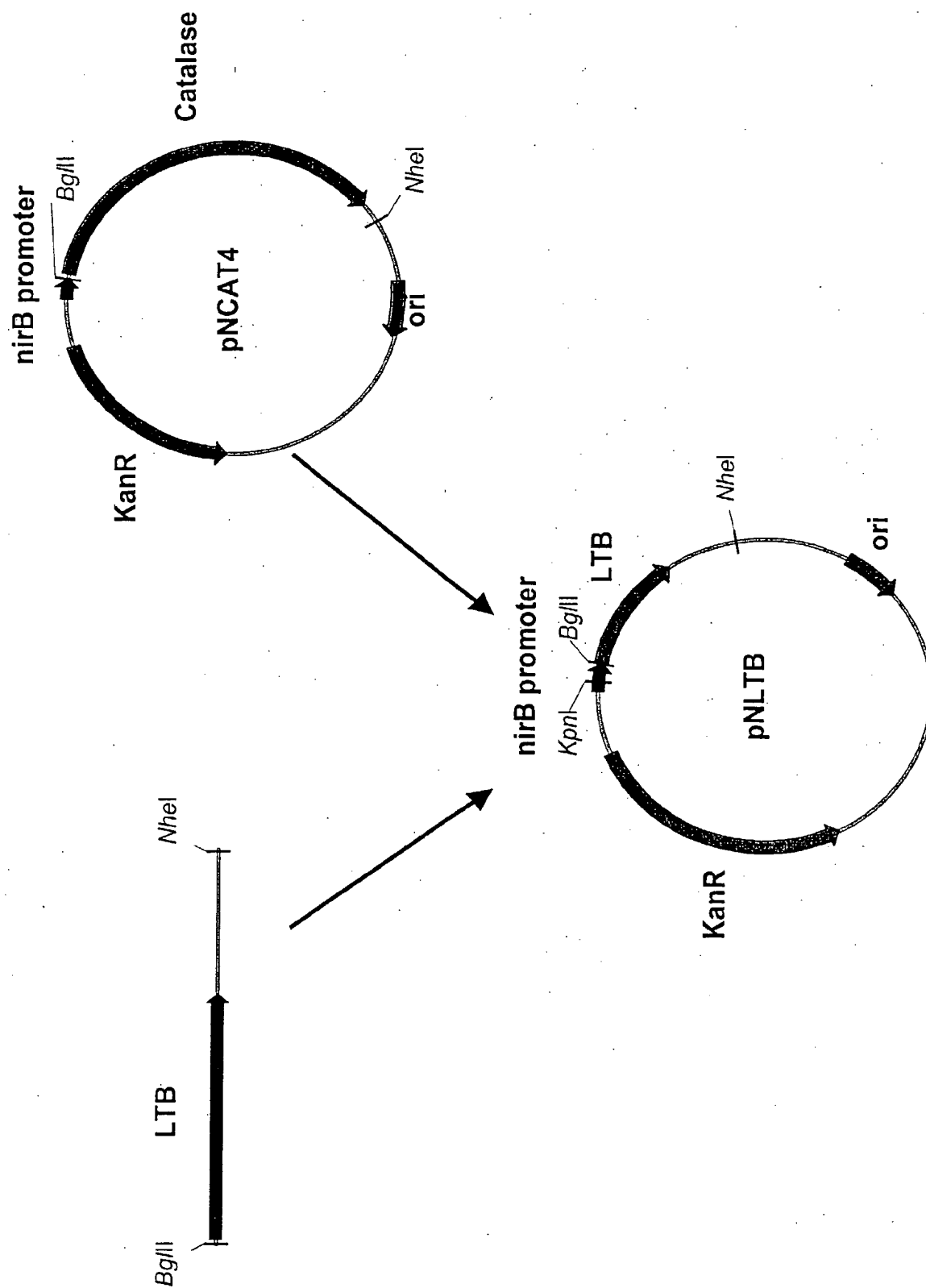
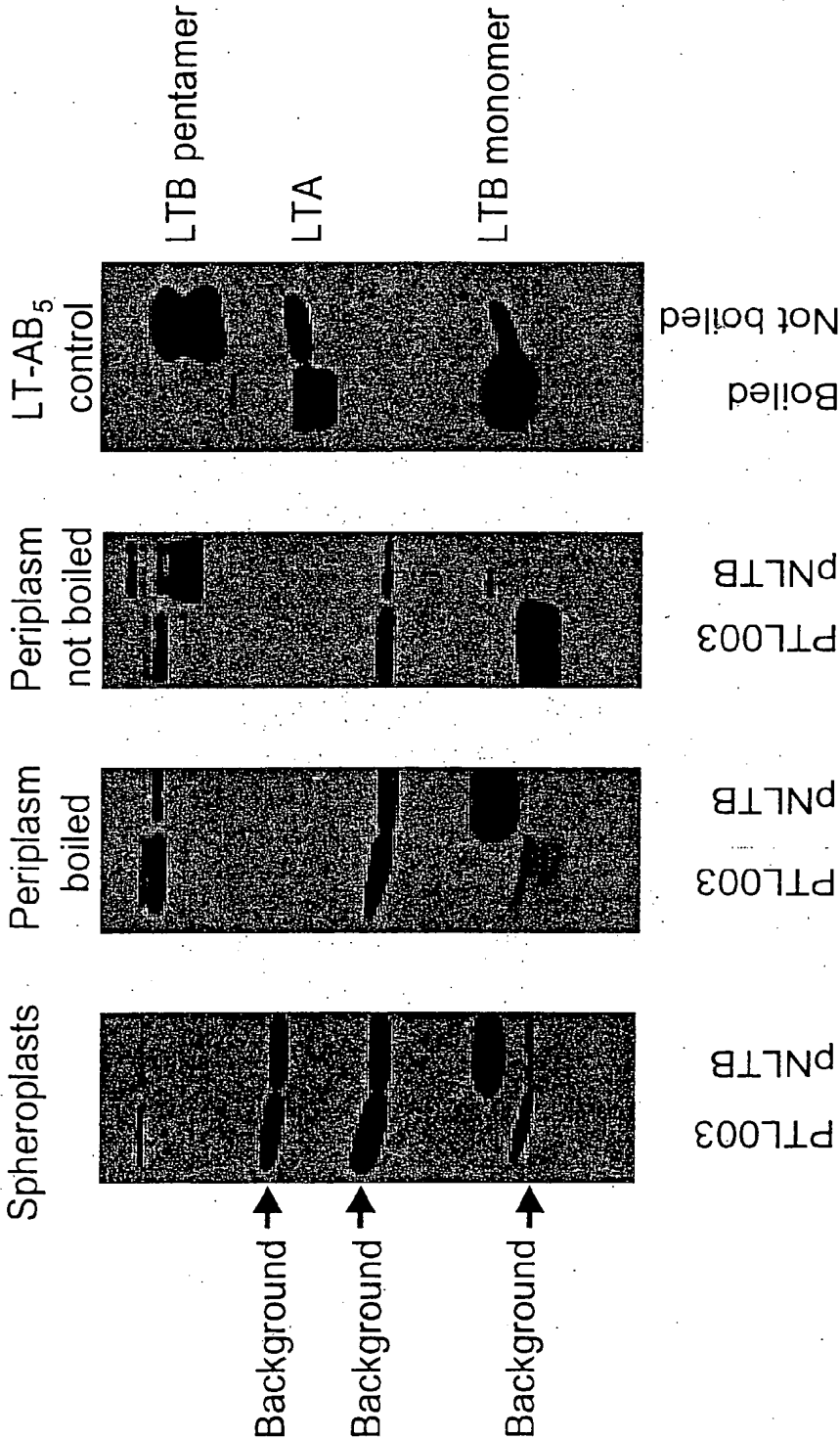


Figure 16



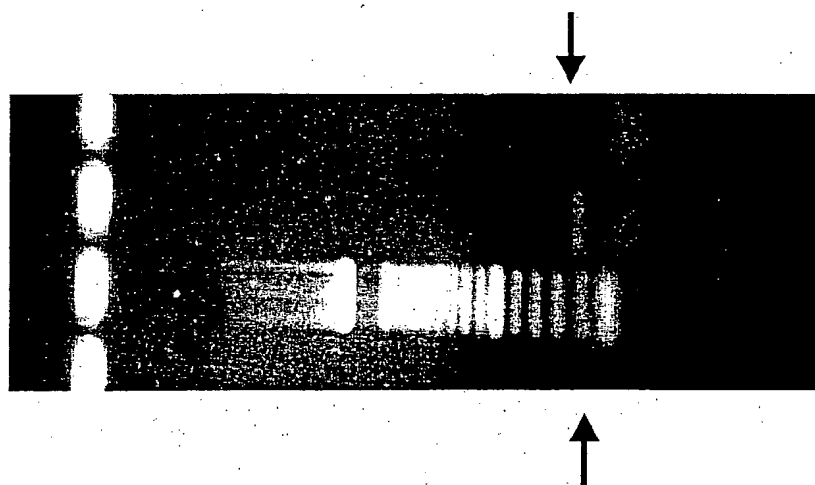
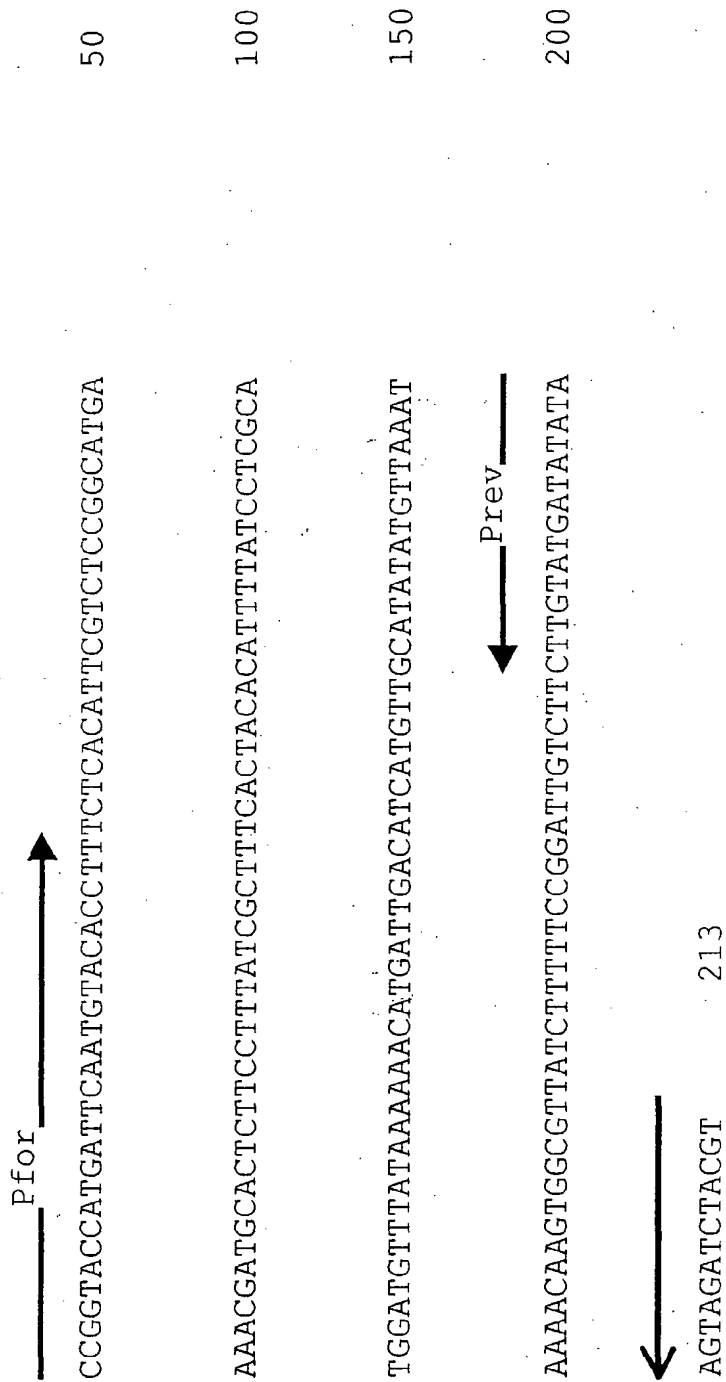


Figure 17

Figure 18



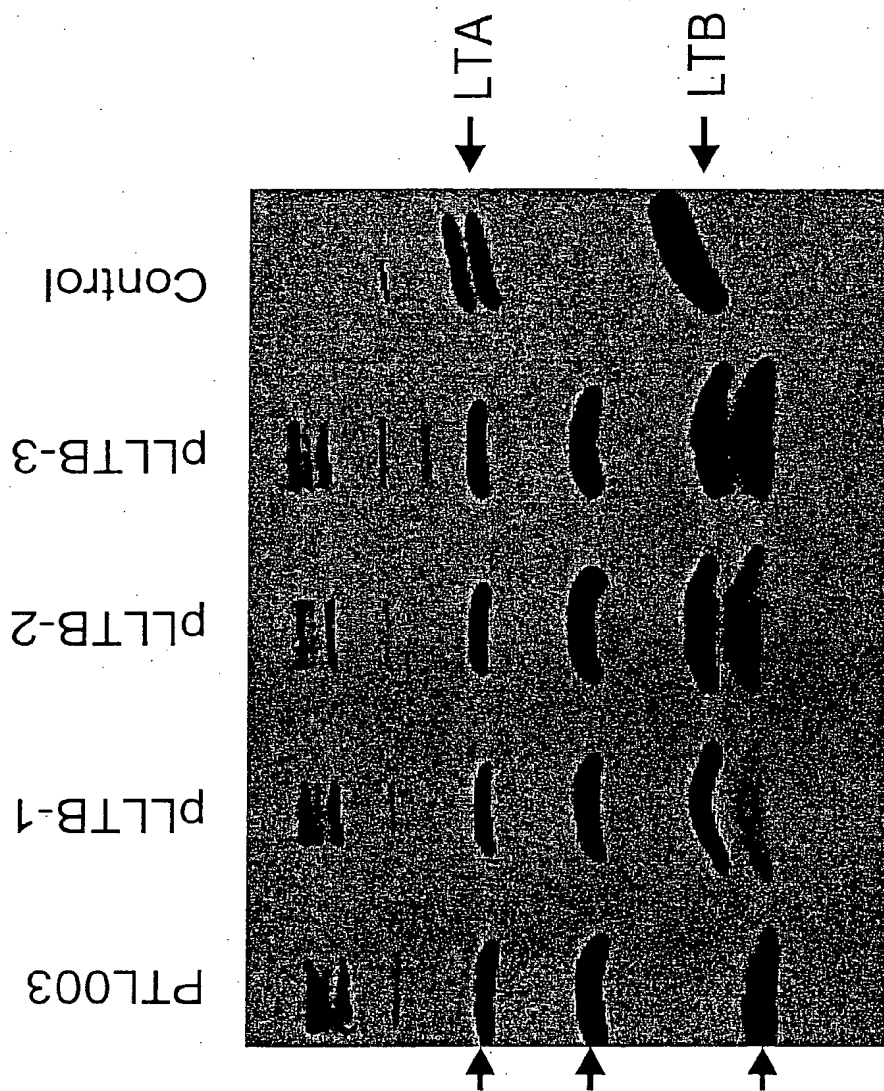
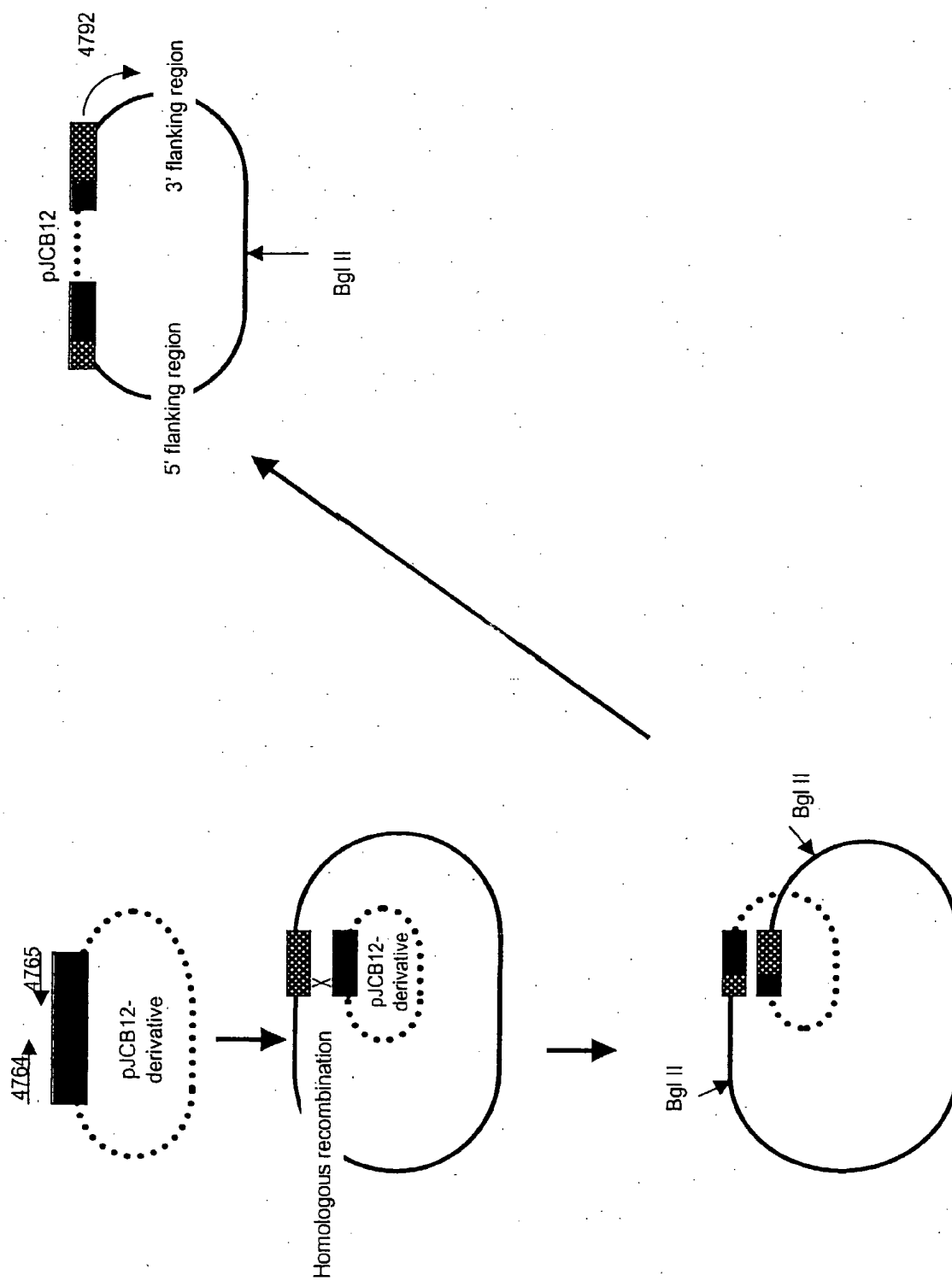


Figure 19

Figure 20



## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 02/04164

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/108 C12N15/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>PANIAGUA M ET AL: "Analysis of incidence of infection with enterotoxigenic Escherichia coli in a prospective cohort study of infant diarrhea in Nicaragua." JOURNAL OF CLINICAL MICROBIOLOGY, vol. 35, no. 6, 1997, pages 1404-1410, XP002221554 ISSN: 0095-1137 page 1407; table 2</p> <p style="text-align: center;">--- -/-</p>	1-6,25

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

\* Special categories of cited documents:

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Date of the actual completion of the international search

28 November 2002

Date of mailing of the international search report

16/12/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Guarinos Viñals, E



## INTERNATIONAL SEARCH REPORT

 Int      onal Application No  
 PCT/GB 02/04164

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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In onal Application No

PCT/GB 02/04164

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TURNER ARTHUR K ET AL: "Construction and characterization of genetically defined aro omp mutants of enterotoxigenic Escherichia coli and preliminary studies of safety and immunogenicity in humans." INFECTION AND IMMUNITY, vol. 69, no. 8, August 2001 (2001-08), pages 4969-4979, XP002221198 ISSN: 0019-9567 page 4970; table 1 page 4973; figure 2 page 4975, right-hand column -left-hand column page 4978, right-hand column	14-19, 26, 28-46,48
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Y	EP 0 087 735 A (TAKEDA CHEMICAL INDUSTRIES LTD) 7 September 1983 (1983-09-07) page 7, line 1 - line 12	20-24
X	BOURGEOIS A L ET AL: "Comparative safety and immunogenicity of two attenuated enterotoxigenic Escherichia coli (ETEC) vaccines in healthy adult volunteers." ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR, vol. 101, 2001, page 344 XP002221199 101st General Meeting of the American Society for Microbiology;Orlando, FL, USA; May 20-24, 2001, <a href="http://www.asmtusa.org/mtgsrc/generalmeeting.htm">http://www.asmtusa.org/mtgsrc/generalmeeting.htm</a> 2001 ISSN: 1060-2011 the whole document	14-17, 19,26, 28-33
X	TURNER A K ET AL: "Construction and characterisation of an attenuated strain of enterotoxigenic E. COLI for use as a live oral vaccine." IMMUNOLOGY LETTERS, vol. 69, no. 1, 15 June 1999 (1999-06-15), page 180 XP001119346 10th International Congress of Mucosal Immunology;Amsterdam, Netherlands; June 27-July 1, 1999 ISSN: 0165-2478 the whole document	14-17, 26,28-33
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## INTERNATIONAL SEARCH REPORT

Int. Patent Application No.  
PCT/GB 02/04164

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	HABERBERGER R L JR ET AL: "Etiology of acute diarrhea among United States embassy personnel and dependents in Cairo, Egypt." AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE, vol. 51, no. 6, 1994, pages 870-874, XP001119246 ISSN: 0002-9637 ---	
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 02/04164

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claim 33 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 02/04164

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